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THE EFFECTS OF VIRUSES ON INTRAOCULAR TISSUES

I. INFECTIONS WITH THE VIRUS OF FOX ENCEPHALITIS (CANINE HEPATITIS)

C. A. EVANS,* MARGARET DOWELL, AND R. G. GREEN†
From the Department of Bacteriology, University of Minnesota, Minneapolis, Minnesota

Histologically, the eye represents a collection of exceptionally diverse kinds of cells of ectodermal and mesodermal origin. As a result of this circumstance, a knowledge of the capacity of different viruses to grow in intraocular tissues is of unusual significance with respect to the tissue specificity of those viruses. In addition, it might be expected that viruses growing in any one or more of the intraocular tissues might induce inflammatory reactions readily visible through the cornea even though the animal exhibited no definite symptoms of general illness. If this were true, the difficulty imposed by the "silent infections" that now constitute a major obstacle to experimental work with many viruses would be overcome in the case of any virus capable of growing in the ocular tissues.

In testing these ideas experimentally, highly successful results were obtained with the first virus studied, that which causes fox encephalitis.[1] However, in subsequent studies we found that some viruses which grew well in intraocular tissues might cause insufficient damage to produce changes readily visible on direct inspection of the eye. Other viruses, if injected in adequate amount, caused a characteristic toxic reaction in the ocular tissues. This reaction, however, was unrelated to infection. In at least one instance it appeared that a combined effect due to infection and toxicity was observed.

In the present paper experiments on the virus of fox encephalitis are described. In subsequent papers findings with the viruses of herpes simplex, infectious feline agranulocytosis, ornithosis, equine encephalomyelitis and influenza, will be described. In addition, negative results obtained in experiments with a number of viruses and with noninfectious materials will be summarized.

The principal objective throughout this study has been to learn about tissue specificity of the viruses under investigation by a detailed study of histopathology and distribution of inclusion bodies in intraocular tissues.

Virus infections of the eye have received relatively little attention, and nearly all of the work done in this field has been concerned with extraocular infections. This is a natural consequence of the comparative rarity of spontaneous intraocular virus infections. A review by Thygeson[2] provides a good summary of studies of extraocular virus infections of man.

Experimental infections of the rabbit cornea with herpes and with viruses of the smallpox group are well known. An interesting series of experiments on intraocular infections with the rickettsia

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* Present address: Dept. of Microbiology, University of Washington School of Medicine, Seattle, Washington.
† Deceased.

1. Evans, C. A., Yanamura, H. Y. and Green, R. G. 1943, Proc. Soc. Exper. Biol. & Med. **53**: 183–186.

2. Thygeson, P. 1943, Arch. Ophth. **29**: 285–300, 488–508, 635–661.

of tsutsugamushi[3–5] yielded results of practical value in the study of this disease. The rickettsia multiplied in endothelial cells* and caused a clouding of the cornea and hyperemia of the iris and pericorneal tissue.

Studies on infection of the eyes of rabbits, guinea pigs and monkeys with the virus of lymphogranuloma venereum have been reviewed by Macnie.[6] There is good evidence that this virus occasionally infects the human eye. Inclusion bodies have been seen in epithelial cells of the human cornea[7] and in endothelial cells from the corneas of guinea pigs.[6]

A number of neurotropic viruses have been injected into the eye as a means of studying spread of the infective agent to and through the central nervous system (CNS). The poliomyelitis virus was found by Burnet et al[8] to infect the CNS of monkeys as regularly after injection into the eye, in the retinal area, as after intracranial inoculation. Sabin and Olitsky[9] briefly noted infection of the retina following intraocular inoculation of the viruses of equine encephalomyelitis and vesicular stomatitis. They found necrosis of the retinal neurones and traced the infection to the contralateral superior colliculus.

Goodpasture and Teague[10] studied the eyes of rabbits following intraocular infection with the virus of herpes simplex. They found inclusion bodies in the ganglion cells of the retina and in glial cells of the optic nerve.

Fox encephalitis infection of the eyes of red foxes

Several years ago it was reported[1] that intraocular inoculation provided a sensitive and experimentally useful means of detecting viable fox encephalitis virus. Experience since that time on hundreds of foxes has confirmed the original impressions as to the utility of this procedure. During the past 5 years this procedure has been employed in the routine preparation and assay of immune serum used in protecting tens of thousands of foxes. This involved the titration of virus and of serum-virus mixtures.

The technique employed varied slightly from time to time. Aqueous humor was first aspirated. Following this, any route of injection of virus into the anterior chamber or into the vitreous humor regularly caused infection of cells lining the anterior chamber.

Evidence of infection is readily observed grossly (fig. 1). The cornea becomes opaque and bluish-gray in color. It is markedly thickened due to edema of the substantia propria. There is little conjunctival exudate or hyperemia. The corneal reaction is not seen during the first day or two but is always fully developed by the fifth day.

To prove that the corneal change was caused by infection with the virus of fox encephalitis, the animal was killed and smears of the endothelial cells lining the inner surface of the cornea prepared.

3. Nagayo, M., Tamiya, T., Mitamura, T. and Sato, K. 1930, Japanese J. Exper. Med. **8**: 309–318.
4. Nagayo, M., Miyagawa, Y. 1931, Japanese J. Exper. Med. **9**: 87–151.
5. Lewthwaite, R. and Savoor, S. R. 1936, Brit. J. Exper. Path. **17**: 448–460.

* In this paper the term "endothelium" is used in referring to the cells lining the inner surface of the cornea, the filtration angle and the anterior surface of the iris. A synonym is "mesenchymal epithelium."

6. Macnie, J. P. 1941, Arch. Ophth. **25**: 255–279.
7. Scheie, H. G., Crandall, A. S. and Henle, W. 1947, J.A.M.A. **135**: 333–339.
8. Burnet, F. M., Jackson, A. V. and Robertson, E. G. 1939, Australian J. Exper. Biol. & M. Sc. **17**: 253–260.
9. Sabin, A. B. and Olitsky, P. K. 1937, Am. J. Path. **13**: 615 (abstract).
10. Goodpasture, E. W. and Teague, O. 1923, J. Med. Res. **44**: 139.

These were stained with any suitable stain, such as hematoxylin and eosin or a modified Schorr's stain,[11] and examined for inclusion bodies. This technique is essentially the same as that used in diagnosing canine distemper except that in the latter disease the inclusion bodies are sought in the epithelium of the bladder or trachea.[12] The inclusion bodies of fox encephalitis are large, intranuclear and readily recognized.

Intraocular infection leads to dissemination of the virus of fox encephalitis throughout the body. Foxes frequently develop the characteristic neurological signs and die of the disease. As with other routes of infection, the incidence of such cases of illness is extremely variable after intraocular inoculation. It is a striking fact that a 10% suspension of infective tissue injected into the brain, or by any route other than intraocular, may cause visible illness in only a moderate proportion of injected foxes, although a 1:1,000,000 dilution of the same material will cause a severe intraocular infection in 100% of injected eyes.

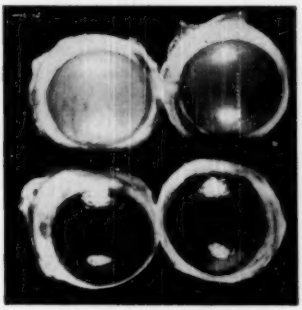

FIG. 1.—Eyes of 2 foxes removed 5 days after intraocular inoculation. The 2 upper eyes were injected with fox encephalitis virus; the 2 lower eyes received the same amount of virus in immune serum. The opacity of the upper eyes is due to marked edema of the cornea. The corneas of the 2 lower eyes remained clear.

Ordinarily inclusion bodies can be found in smears of endothelial cells from only those eyes exhibiting the characteristic corneal opacity. In a few instances small numbers of inclusions have been found in smears from normal-appearing eyes 5 days after injection with very small amounts of virus. Apparently an inoculum of 2 or 3 times the absolute minimal infective dose is sufficient to cause an intraocular infection that is as severe as that caused by much larger amounts of virus.

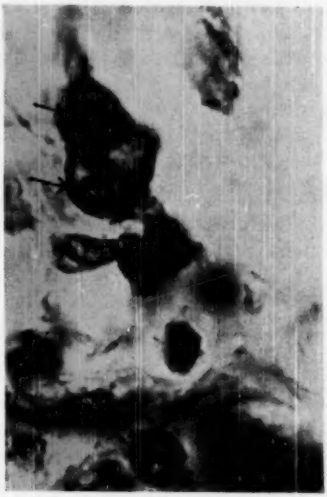

FIG. 2.—Inclusion bodies of fox encephalitis in cells of filtration angle. In infected eyes like the two shown in figure 1, inclusion bodies of this sort are numerous in infected endothelial cells lining the inner surface of the cornea as well as in certain other cells.

Fox encephalitis infection in the eyes of dogs

Several years ago Green and his associates described in some detail the in-

11. Page, W. G. and Green, R. G. 1942, Cornell Vet. **32**: 265.
12. Green, R. G. and Evans, C. A. 1939, Cornell Vet. **29**: 35.

fection caused by the virus of fox encephalitis in dogs.[13] The term "fox encephalitis" has become well established in the veterinary literature even though the virus is known to infect dogs. There is little doubt that, as Rubarth[14] indicates, the disease described in recent years as canine hepatitis is caused by the same virus. In dogs this virus as a rule causes much more hepatic injury than is seen in foxes.

Inoculation of the fox encephalitis virus into the eyes of dogs causes the same type of reaction as that seen in foxes. The cornea becomes opaque and thickened with edema; inclusion bodies are found in smears of endothelial cells. The reaction develops at the same rate, being fully developed on the fifth day. As in the case of foxes, some animals develop signs of generalized infection and may die. Many show no such evidence of infection outside of the eye.

In one instance tests were made for virus in liver and spleen of a dog that had an intraocular infection without evidence of illness outside of the eye. The right and left eyes of this dog, K549, were injected on January 8, 1946 with 10^{-2} and 10^{-3} dilutions of a preparation of stock virus. At 3 days, both corneas were opaque. At 6 days, the animal showed no evidence of infection other than the ocular changes. At this time the dog was killed. Dilutions of 10^{-1} and 10^{-3} of its liver and spleen were injected into the 4 eyes of 2 normal dogs. All 4 eyes developed the characteristic opacity, and inclusion bodies were found in all 4 eyes.

These results show that in animals not exhibiting symptoms of extraocular disease, the virus may be widely distributed. It has previously been shown[1] that virus may grow in lymph nodes of foxes undergoing an inapparent infection. Foxes and dogs appear to be about equally susceptible to intraocular infection with the virus of fox encephalitis. This impression has been gained in experiments with animals of the two species over a period of many years.

In table 1 (exp. of Feb. 3, 1945), the results of an experiment made on this point are presented. It is shown that the virus used in this experiment caused a typical severe infection in both fox and dog eyes in a dilution of 10^{-6}. Virus diluted to 10^{-7} caused an atypical limited infection in the two eyes of one fox and no infection in the one dog eye injected.

TABLE 1.—*Comparative susceptibility of dogs and foxes to intraocular infection with fox encephalitis virus.*

	Dogs							
	K356		K357		K358		K359	
Eye	R	L	R	L	R	L	R	L
Concentration of inoculum	10^{-2}	10^{-4}	10^{-5}	10^{-6}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Results								
a. Opacity of cornea	++++	++++	++	Pus	++++	+++	0	0
b. Corneal inclusions	++++	++++	++++		+++	+++	0	0

	Foxes							
	K352		K353		K354		K355	
Eye	R	L	R	L	R	L	R	L
Concentration of inoculum	10^{-2}	10^{-4}	10^{-5}	10^{-6}	10^{-6}	10^{-7}	10^{-7}	10^{-8}
Results								
a. Opacity of cornea	++++	++++	+++	+	+++	0	0	0
b. Corneal inclusions	++++	+++	++++	++++	++++	+ +	5 inclusions	0

Inoculum was pooled fox brain diluted as shown in table from a stock suspension of 20% brain tissue in 50% neutral glycerine.

13. Green, R. G. and Shillinger, J. E. 1934, Am. j. Hyg. **19:** 362–391.
14. Rubarth, S. 1947, Acta path. et microbiol. Scandinav., Supplementum LXIX.

Fox encephalitis infection in the eyes of raccoons

Over a period of several years, Green and his associates used 28 raccoons in attempts to infect animals of this species with the virus of fox encephalitis by the intracranial and intramuscular routes. These experiments yielded consistently negative results. Animals of this species seemed to possess a natural immunity to the disease. Subsequently it was demonstrated[15] that raccoons are highly susceptible to intraocular infection with this virus. After injection of the virus into the eye, the characteristic opacity of the cornea develops with the same severity and regularity as in foxes and dogs. Inclusion bodies are abundant in smears of corneal endothelium.

Fox encephalitis virus has been maintained more than 10 serial passages by intraocular inoculation in raccoons. Occasional animals develop convulsions or other neurological symptoms but this is seen much less frequently than in dogs or foxes. We have no reason to believe that the endothelial cells of the raccoon eye are any less susceptible to the virus than those of the fox or dog eye. Why the infection is frequently lethal in canines but nearly always asymptomatic in raccoons, we do not know.

Fox encephalitis infection of the eyes of cats

In a previous paper[16] it was reported that house cats were resistant to fox encephalitis. Kittens injected subcutaneously and intramuscularly with relatively large doses of virus showed no signs of illness.

Intraocular inoculation of virus has shown that tissues of cats possess a limited susceptibility to the virus of fox encephalitis. Since 1944, 21 house cats of various ages have been injected intraocularly with the virus of fox encephalitis. In no instance did we see a typical severe opacity of the cornea of the type seen regularly in dogs, foxes and raccoons. In one eye of one kitten, K196, a moderate haziness of the cornea developed, and endothelial cells from this cornea contained moderate numbers of inclusion bodies. The other eye of this kitten was injected with the same amount of virus. A definite opacity covering about half of the cornea developed. Inclusion bodies were abundant in the endothelial cells from the latter cornea and somewhat fewer in cells from the eye showing the lesser degree of cloudiness.

In no other instance did we see a definite change in the transparency of the cornea. In 2 other cats typical fox encephalitis inclusions were found in eyes that had been inoculated with virus 6 days previously. In one of the experiments kittens were observed for 9 to 11 days, but all were negative both grossly and microscopically.

In no instance has an inoculum less concentrated than a 10^{-3} dilution of tissue been effective in producing infection even though the same material caused infections in dogs or foxes when diluted 10^{-6} or 10^{-7}.

Failure to infect the eyes of opossums and rabbits with the virus of fox encephalitis

In 1943, 14 rabbits were inoculated intraocularly with fox encephalitis virus. Two strains of virus were injected into the eyes of 2 groups of 7 rabbits each. One rabbit from each group was killed at 1, 2, 3, 4, 5, 7 and 10 days. The eyes were examined histologically. Both gross

15. Green, R. G., Evans, C. A. and Yanamura, H. Y. 1943, Proc. Soc. Exper. Biol. & Med. **53**: 186.
16. Green, R. G., Ziegler, N. R., Carlson, W. E., Shillinger, J. E., Tyler, S. H. and Dewey, E. T. 1934, Am. J. Hyg. **19**: 343–361.

TABLE 2.—*Distribution of inclusion bodies in eyes of foxes and raccoons after intraocular injection of fox encephalitis virus.*

Animal	Time in days*	Route of inoculation	Cornea			Iris						Filtration angle			Ciliary body					Ora serratia		Visual retina					Choroid coat		
			Stratified squamous epithelium	Substantia propria	Endothelium	Endothelium	Fibroblasts	Chromatophores	Radial muscle	Circular muscle	Retinal epithelium	Endothelium	Fibroblasts	Mononuclears	Nonpigment epithelium	Pigmented epithelium	Chromatophores	Fibroblasts	Endothelium	Nonpigment epithelium	Pigmented epithelium	Ganglion cells	Bipolar cells	Rods and cones	Pigmented retina *	Endothelial cells	Fibroblasts	Endothelial cells	Chromatophores
Raccoons																													
30845			0	0	++++	+	?	?	0	0	0	++	+++	++	0	0	0	0		0	0	0	0	0	0				?
30846			0	0	0	0	?	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	?		?	++	
30847			0	+	++++	0	+	0	0	0	?	0	0	0	0	0	0	0		0	0	0	0	0	0		0	0	0
30848			0	+	++++	?	+	0	0	0	0	++	++	++	0	0	+	+	0	0	0	0	0	0	0		0	+	0
30849			0	0	+++	+	?	0	0	0	?	++	++	++	0	0	0	+	0	0	0	0	0	0	0		0	0	0
Foxes																													
K181	4	a.c.	0	+	+++	++	?	+	0	0	?	+++	++	+++	+	?	?	++	0	0	0	0	0	0	0	+	0	+	0
K184	3	Vit.	0	+	++++	++	+		0	0	0	++	++	++	0	0	0	+	0	0	0	?	0	0	0		+	++	0
K185	5	Vit.	0	+	+++	++	+	0	0	0	0	++	++	++	0	0	0	0	0	?	0	0	0	0	0	+	0	0	0
K188	3	c.b.	0	0	+++	++	+	?	0	0	0	++	++	++	0	0	0	+	0	0	0	0	0	0	0		?	0	?
K189	5	c.b.	0	+	+++	++	+	0	0	0	0	++	++	++	0	0	0	0	0	0	0	0	0	0	+		0	0	0

a.c. =anterior chamber. Vit. =into the vitreous close to the retina. c.b. =on the inner surface of the ciliary body.
Inclusions in substantia propria of cornea were nearly all near limbus.
? =Identity of the kind of cell was not positive or there was some doubt about the inclusion.
* Time from inoculation to death of the animal.

and microscopic studies were negative for evidence of infection.

In a number of experiments opossums were injected with fox encephalitis virus by various routes, including the intraocular route. In no animal did we see evidence of infection; no inclusion bodies were found. As a marsupial, the opossum bears its young at a very early stage of development and harbors them in its pouch. As a result of this circumstance very young opossums analogous to fetal stages of other animals are readily available for experimentation. Such animals proved fully resistant to the virus of fox encephalitis.

Nature of the resistance of rabbits, opossums and cats

It has been reported that the serum of some animals is virucidal for certain viruses to which that animal has a natural resistance. For example, Koprowski[17] has demonstrated a nonspecific inactivation of several viruses by serum from some species of rodents and marsupials.

An experiment was made to test the serum of rabbits, opossums and cats for their capacity to neutralize the virus of fox encephalitis. The virus used in this test represented a 10^{-4} and a 10^{-5} dilution of spleen from an infected dog. A simultaneous control titration of the virus in dog eyes showed that 10^{-6} represented the minimal infective dose.

Serum from two opossums was pooled, serum from two cats was pooled and serum from a single rabbit was tested separately. Equal volumes of serum from the three species were mixed with appropriate dilutions of virus, and the mixtures were incubated one hour at room temperature. Each mixture was then injected into 2 eyes of dogs. Therefore, 4 eyes were injected with mixtures containing each of the 3 kinds of serum tested. Of the 12 eyes injected, 11 showed definite infection. One eye injected with a mixture of opossum serum and a 10^{-5} dilution of virus was negative. The 3 other eyes included in the test of opossum serum were all positive.

From these results it is concluded that the resistance of rabbits, opossums and cats to fox encephalitis virus is not based on virucidal activity of the serum.

Distribution of inclusion bodies in the intraocular tissues of animals infected with fox encephalitis virus

An important consideration in any study of intraocular virus infections is the marked diversity of cell types that can be directly exposed to a single inoculum. The visual retina contains a wide range of cells of neural origin; the iris includes epithelium of neural origin on its posterior surface, endothelium of mesodermal origin on its anterior surface and many different kinds of cells in its stroma. The muscle of the iris is peculiar in that it is considered to be derived from ectoderm. Similarly the choroid, the ciliary body and the cornea contain many significantly different kinds of cells. An inoculum of virus deposited in the anterior chamber by way of a needle inserted through the cornea, or through the conjunctiva, sclera and ciliary body, rapidly comes into contact with all of the cells lining the anterior chamber. In addition, as can be seen from table 2, the virus spreads to the stroma of the retina, ciliary body, choroid and visual retina.

A careful mapping of the distribution of inclusion bodies in the inoculated eyes of 5 raccoons and 5 foxes has been made. The results are presented in table 2. From this study, no significant difference between the infections in the eyes of raccoons and foxes is apparent. One raccoon, 30846, apparently had a

17. Koprowski, H. 1946, J. Immunol. **54**: 387–394.

minimal infection limited to the sclera and choroid. Except for this animal, all had numerous inclusion bodies in the endothelial cells on the inner surface of the cornea. Similarly inclusion bodies were regularly found in endothelial cells of the surface of the iris and the filtration angle. Some cells in the stroma of the cornea were infected in most animals. In 3 of the 10 animals inclusions were present in small numbers in the sclera.

In the iris, inclusions were found chiefly in endothelial cells and fibroblasts. They were not found in muscle cells. In one instance a clearly recognizable inclusion body was found in a chromatophore. In two other eyes chromatophores considered as containing "probable inclusions" were found. Pigment made it especially difficult to study these cells satisfactorily. Careful study of the retinal epithelial cells on the posterior surface of the iris was made, and in three instances it appeared that cells of this type containing inclusion bodies were found. However, the pigment of these cells, as in the chromatophores, seriously interfered with examination of the cells for inclusions.

In the filtration angle, endothelial cells, fibroblasts and mononuclear cells (presumably macrophages) were regularly infected.

The ciliary body is in some respects analogous to the iris. However, from the small numbers of inclusion bodies in endothelial cells and fibroblasts, its stroma seemed to have been sparingly exposed to virus. Therefore, in this area the negative findings were considered less significant than the positive results. In one animal, K181, an inclusion body was identified in a cell of the nonpigmented epithelium of the ciliary body. In no instance was an inclusion body identified beyond question in a chromatophore or in a cell of the pigmented epithelium of this structure.

In the visual retina the most significant finding was a well developed inclusion body in a cell of the pigmented epithelium layer. Cells of this layer do not have pigment in the central areas farthest from the ciliary body. It was in this area, in a cell lacking pigment, that the inclusion was found. In two eyes inclusion bodies were found in endothelial cells of small vessels of the retina. A single supporting cell of the rod and cone layer contained a structure that was considered as probably an inclusion body.

In the choroid coat, occasional fibroblasts and endotheliai cells were infected.

Histotropism of fox encephalitis

In previous studies[18] the susceptibility of various tissues of susceptible animals to fox encephalitis has been extensively explored. A definite pattern of susceptible kinds of cells has been shown to exist.

The significance of the characteristic inclusion bodies as indicators of cellular infection is well supported by their occurrence in a number of tissues only after direct inoculation. That the absence of inclusion bodies in a cell indicates freedom of that cell from infection is more difficult to prove. A number of negative transmission experiments would be required for every tissue under consideration in each species of animal. The fact that no pathological changes have been detected in tissues lacking inclusion bodies is of some significance. If may be further stated that to our knowledge no one has shown that any virus capable of producing large intranuclear inclusion bodies with margination of chromatin ever infects cells without producing such inclusion bodies. In

18. Green, R. G. and Evans, C. A. 1939, Am. J. Hyg. **29**: 73.

this paper it has been assumed, as a working hypothesis, that those tissues lacking inclusion bodies are free of infection.

Endothelial cells are highly susceptible as shown by the frequent occurrence of inclusion bodies in cells lining blood vessels of the brain, capillaries of glomeruli and in peripheral sinuses of lymph nodes draining an area of infection. The related cells of the reticulo-endothelial system are equally susceptible as indicated by inclusion bodies occurring in Kupffer cells of the liver and histiocytes in various locations. Fibroblasts in capsules of infected lymph nodes or elsewhere are also frequently infected.

In the eye, the susceptibility of endothelial and reticulo-endothelial cells and fibroblasts was as great as expected from studies in other parts of the body. The characteristic and useful corneal reaction to infection, opacity and edema is a direct result of infection of the corneal endothelium with consequent disturbance of the semipermeable membrane which normally separates the stroma of the cornea from the aqueous humor.

In addition to the endothelial and reticulo-endothelial cells, fox encephalitis virus attacks certain specific cells of epithelial type as follows: (1) squamous epithelium over the tonsil, but not elsewhere,[19] (2) ependyma lining the ventricles but not that which covers the choroid plexus,[20] (3) hepatic cord cells of dogs[21] and, to a much lesser extent, of foxes, and (4) infrequently, adrenal cortical cells.[21]

19. Green, R. G., Green, B. B., Carlson, W. E. and Shillinger, J. E. 1936, Am. J. Hyg. **24**: 57–70.
20. Green, R. G., Katter, M. S., Shillinger, J. E. and Hanson, K. B. 1933, Am. J. Hyg. **18**: 462–481.
21. Green, R. G. and Shillinger, J. E. 1934, Am. J. Hyg. **19**: 362–391.

The susceptibility of the cells listed above is in striking contrast to the complete resistance of other types of epithelium. In studies of many hundreds of animals, inclusion bodies have never been found in glandular cells other than those of the liver. In routine studies of thyroid, salivary glands, pancreas (acini and islands of Langerhans) and gastric mucosa from scores of animals infected with fox encephalitis, not one inclusion body has been found in a secretory cell. Similarly, surface epithelium, with the exception of squamous cells over the tonsil and ependyma of the ventricles, has always been free of inclusion bodies. Our routine studies have included bronchial mucosa and mucosa of large and small intestine; pelvis of the kidney, and urinary bladder; and ducts of salivary glands, pancreas and liver.

We were interested to determine whether, in the eye, epithelial cells of neural origin would have a susceptibility comparable to ependymal cells or a resistance comparable to other cells of neural origin. It was found that the cuboidal epithelium that covers the iris, ciliary body and choroid coat exhibited only the very lowest degree of susceptibility. In only two cells were inclusions positively identified, and in five other cells were structures considered as "possible" or "probable" inclusions found. Although the pigment in many of these cells interfered with good visualization, it is felt that the general conclusion that cells of this type exhibit a susceptibility that is quantitatively different from that of ependymal cells is well justified.

A puzzling and undoubtedly significant question concerns the nature of the differences in susceptibility observed between (1) hepatic cord cells of dogs (highly susceptible) and all other secretory cells studied (completely resistant), (2) tonsilar epithelium (high

susceptibility) and all other surface epithelium (completely resistant), (3) ependyma of ventricles (high susceptibility) and ependyma of choroid plexus (complete resistance) and retinal epithelium (lowest detectable susceptibility).

It should be noted that although many viruses are present in the blood stream, relatively few have been known to infect the vascular system. Rickettsia, on the contrary, are noted for their capacity to infect endothelial cells. Whether this similarity in the histotropism of fox encephalitis virus to that of rickettsia reflects an evolutionary relationship or is merely coincidental cannot be determined on the basis of information presently available.

Species susceptibility to fox encephalitis

Extensive studies of the susceptibility of various kinds of animals to the virus of fox encephalitis by Green and his associates[22] before the intraocular method of infection was used, defined the susceptible animals as those species included in the family Canidae, except the gray fox. Red foxes, dogs and coyotes were susceptible. The gray fox, a relatively primitive type as compared with other species tested, was resistant. Similarly raccoons, cats, ferrets, sheep, monkeys, white rats, guinea pigs, gray squirrels and rabbits were all shown to be resistant.

With introduction of the intraocular method of inoculation, a practical method became available of testing for growth of virus independently of symptoms of a general illness. On this basis the virus was shown to grow abundantly in the tissues of raccoons which belong to the family Procyonidae, and to grow to a limited extent in tissues of house cats, of the family Felidae. All of these animals are in the order Carnivora. Rabbits and opossums which fall into quite different taxonomic groups of mammals were completely resistant to the virus of fox encephalitis.

The range of suceptible animals, or the zootropism of the fox encephalitis virus, thus is seen to be considerably extended by the intraocular method of inoculation. However, the striking conformity to general taxonomic lines is maintained.

The species specificities of the great majority of viruses fall into one of two patterns. Some infect animals of two or more families without regard for zoologic similarities of those families. Others are highly restricted, infecting animals of one species, one genus or at most one family.*

Influenza virus is a good example of the former type. It grows best in ferrets, white mice and men, three species of entirely different zoologic groups. Outstanding among the viruses with a highly restricted zootropism is the whole group of proliferative viruses. The viruses of Shope papilloma, canine oral papilloma, rabbit myxoma, human verrucca vulgaris and Rous sarcoma infect only a single species, or at most closely related species. Other viruses, measles for example, are highly adapted to one species and show little virulence for unrelated species.

Canine distemper and fox encephalitis viruses are unusual in that both will infect animals of several zoologic families, but these families are closely related; they belong to the same order, Carnivora. It is noteworthy that the zootropism of canine distemper includes all animals susceptible to fox en-

22. Green, R. G., Ziegler, N. R. Carlson, W. E., Shillinger, J. E., Tyler, S. H. and Dewey, E. T. 1934, Am. J. Hyg. **19**: 343–361.

* The susceptibility of embryonic tissue is known to cross the lines drawn on the basis of susceptibility of tissues in adult animals. This constitutes a separate problem.

cephalitis and in addition the Mustelidae. The gray fox, the one canine species known to be resistant to fox encephalitis, is susceptible to distemper.

SUMMARY

In a study of intraocular infections with the virus of fox encephalitis or canine hepatitis it was found that certain tissues in the eyes of red foxes, dogs and raccoons were highly susceptible. The corresponding tissues in the eyes of cats were infected only with relatively large doses of virus and frequently failed to become infected even with large doses. When infection did occur, inclusion bodies were found in fewer cells, and the macroscopically visible reaction was less severe than in eyes of foxes, dogs, or raccoons.

Rabbits and opossums were completely resistant. Serum from normal animals of these species and from normal cats failed to inactivate the fox encephalitis virus.

The intranuclear inclusions of fox encephalitis are characteristic and are considered to signify virus infection of the cells in which they are found. Detailed histopathologic studies of infected eyes of foxes and raccoons showed no significant difference in the distribution of inclusion bodies in the two species. Relatively large numbers of inclusions were found in endothelial cells lining the anterior chamber (inner surface of cornea, filtration angle and iris), in fibroblasts and in macrophages. Cells lacking inclusion bodies and therefore considered resistant to the virus included all elements of the retina of neural origin (including pigmented and nonpigmented epithelium and muscle of the iris) and chromatophores. Exceptions to this resistant state occurred in the case of three cells containing typical inclusion bodies, one chromatophore, one cell of the nonpigmented epithelium of the ciliary body and one cell of the pigmented epithelial layer outside the visual retina.

The similarity of the histotropism of the fox encephalitis virus to that of many rickettsia is pointed out. The species specificity of fox encephalitis virus is discussed.

THE RELATIONSHIP OF PROTEIN DEPLETION TO NATURAL RESISTANCE

C. HAROLD STEFFEE

From the Department of Pathology, The University of Chicago, Chicago 37, Illinois

Many investigators have attempted to explain the phenomenon of "natural resistance," whereby a given animal species is relatively insusceptible to infection with a pathogenic microorganism. This resistance, in contrast to acquired immunity, occurs without previous exposure to the microorganism and without the development of specific antibodies. Metchnikoff and his pupils attempted to demonstrate immune substances in the serum of resistant animals and accomplished this for some but not for all animal species or all bacteria tested. Later, specific opsonins were found in the serum of pneumococcal-resistant animals, such as swine, sheep, and chickens,[1] and in dogs and cats[2] which had never been infected with pneumococci. Humoral antipneumococcal substances have been demonstrated in vitro and in vivo, but in the latter instance, highly controversial results have been obtained. Kyes and others have ascribed natural resistance to the protective action of the phagocytic cells. In the pigeon pneumococci injected intravenously or intraperitoneally are rapidly ingested by the Kupffer cells of the liver and the macrophages of the spleen and bone marrow.[3,4] A similar reaction has been demonstrated in dogs[5] and in immunized animals.[6] But it seems difficult to understand why certain species of animals should actively phagocytose some bacteria and not others, unless the bacteria are agglutinated or in some way rendered more susceptible to phagocytosis. The importance of humoral substances and of phagocytosis has been minimized by other workers, who have suggested body temperature as the primary mechanism of defense. Again, controversial results have been obtained by various investigators.

The studies of the effects of nutritional deficiencies upon natural resistance have been related chiefly to vitamin deficiency. The divergent results obtained have been reviewed by several authors.[7–10] In general, the effects of vitamin deficiencies have been most conspicuous when the dietary restrictions were great enough to result in a weight loss.[11,12] This is usually accom-

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The work has been aided, also, by The National Livestock and Meat Board, and The Douglas Smith Foundation for Medical Research of the University of Chicago.

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1. Robertson, O. H. and Sia, R. H. P. 1927, J. Exper. Med. **46:** 239–262.
2. Terrell, E. E. 1930, J. Exper. Med. **51:** 425–440.
3. Kyes, P. 1916, J. Infect. Dis. **18:** 277–292.
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6. Cannon, P. R., Sullivan, F. L. and Neckerman, E. F. 1932, J. Exper. Med. **55:** 121–137.
7. Clausen, S. W. 1934, Physiol. Rev. **14:** 309–350.
8. Robertson, E. C. 1934, Medicine, **13:** 123–206.
9. Perla, D. and Marmorsten, J. 1941, Natural Resistance and Clinical Medicine, Boston, Little, Brown and Company.
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11. Werkman, C. H. 1923, J. Infect. Dis. **32:** 255–262.
12. McCarrison, R. 1918, Indian J. M. Res. **6:** 339–355.

panied by a loss of appetite, which may result in multiple dietary deficiencies, including protein insufficiency, which may be further aggravated by derangements of internal metabolism resulting from the vitamin restriction.

There have been few conclusive studies on the effect of protein starvation on natural resistance. For example, Jägerroos kept two dogs on low protein diets for periods of 7 and 11 months, respectively, and noted that both of them developed spontaneous terminal infections.[13] Thomas demonstrated that suckling pigs resisted tuberculous infection better when they were given a diet rich in protein.[14] Sako showed that the survival time of mice infected intraperitoneally with pneumococci was reduced when the mice were given a low protein diet and was augmented by feeding diets rich in protein.[15] These differences were uniform, and they were statistically significant. The resistance of mice to *Salmonella enteriditis* has been investigated.[16,17] However, these workers discard all mice dying within 48 hours and publish only the mortality figure obtained after 30 days. By this procedure they have combined natural resistance with the ability of the animal to produce antibodies during the course of an infection. Thus these experiments permit no conclusions concerning the effect of diet upon *natural* resistance.[18] Rats fed a low protein diet have been shown to be more susceptible to oral sulfanilamide[19] and to parenteral diphtheria toxin[20] and tetanous toxin.[21] In a previous paper I have demonstrated that pigeons lose their natural resistance to pneumococci after prolonged protein starvation.[22] In addition, Woolridge has investigated the effect of protein depletion upon natural and acquired immunity to the Friedländer bacillus. He has shown that natural resistance, as measured by the mortality rate of infected protein-depleted and normal rats within 48 hours after the time of injection, is reduced by protein insufficiency.[23] To my knowledge the only way in which protein depletion has been shown to affect natural resistance is by the reduction of the phagocytic activity of leucocytes.[24,25]

In view of the controversial reports on the fundamental mechanisms of natural resistance, further investigation of the problem is obviously needed. Protein depletion is known to reduce body temperature and to depress phagocytic activity. Further, if the opsonins, mouse-protecting substances, and bacteriostatic substances are proteins, a prolonged period of protein starvation might reduce their concentration and thus decrease natural resistance. Accordingly, these studies were carried out in protein-depleted and normal roosters, since the normal cockerel is highly resistant to pneumococcal infection. Preliminary experiments with full-grown birds demonstrated that 6 or 7 months were necessary to produce severe pro-

13. Jägerroos, B. H. 1902, Skandinav. Arch. f. Physiol. **13**: 375–418.
14. Thomas, E. 1919, Ztschr. f. Kinderh. **24**: 235–280.
15. Sako, W. S. 1942, J. Pediat. **20**: 475–483.
16. Schneider, H. A. and Webster, L. T. 1945, J. Exper. Med. **81**: 359–384.
17. Schneider, H. A. 1946, J. Exper. Med. **84**: 305–322.
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21. Csáky, P. C. 1943, Arch. f. d. ges. Physiol. **247**: 53–62.
22. Steffee, C. H. 1946, Unpublished Master's thesis, Department of Pathology, University of Chicago.
23. Woolridge, R. L. 1949, Federation Proc. **8**: 376.
24. Mills, C. A. and Cottingham, E. 1943, J. Immunol. **47**: 503–504.
25. Wissler, R. W. 1947, J. Infect. Dis. **80**: 264–277.

tein depletion, and even then there was marked variability within the group. Subsequently, more uniform depletion was obtained by feeding a group of birds a low protein diet when they were about 2 months old. Under these conditions, the most severely depleted birds were still large enough to furnish serum for the mouse-protection studies without precipitating hemorrhagic shock. Since the liver, spleen, and marrow are the major sites of phagocytosis, the organisms were given intravenously.

MATERIALS AND METHODS

Birds.—Pullorum-tested, day-old, single comb white Leghorn cockerels were obtained from various poultry houses, and were kept in individual wire-bottomed cages. For each experiment all birds were of the same age and were obtained from the same source. Food and water were supplied in silos which were hung on the sides of the cages and were rarely fouled. In addition the birds had access to crushed oyster shell.

Diets.—The chicks were given a commercial starting mash ("Kay-Bee" starting mash, obtained from Kahn Brothers, Chicago) until they were 6 weeks old, after which they were given a growing mash. When the cockerels weighed 675 to 750 g, they were started on 150 g per day of ration C. After 2 weeks, they were fasted for 12 hours, weighed, and divided into groups in the following manner. Two birds of identical or similar weights were chosen. The bird which had the greater consumption of diet C for the preceding 2 weeks was given 150 g per day of diet D_2; the other bird continued receiving the same amount of diet C. By this process the starting weights of the two groups were equalized, and the inappetence of the birds given diet D was counteracted. Diet consumption was carefully determined each day for the first three experiments. The wasted food, collected beneath the floor screens of the cages, was weighed three times a week and subtracted. The composition of these diets is given in table 1. The protein content of diet C was calculated to approximate the amino acid requirements for growth of the chick. Comparison with the data collected by Almquist[26] demonstrates that the objective was accomplished, but that ration C contained a relative excess of leucine and isoleucine. The birds receiving this ration grew satisfactorily. After three months, their weight gain was comparable to that obtained by feeding commercial growing mash ad lib.

TABLE 1.—*Composition of control and low protein rations (per kg).*

Diet constituents	Units	Control (C)	Low protein (D)
Casein (SMACO-vit. test)	g	140	0
Gelatin	g	80	0
Dextrin	g	303.5	413.5
Cornstarch	g	303.5	413.5
Corn oil	g	37.5	37.5
Wheat germ oil*	g	2.5	2.5
Ruffex (Fisher)	g	35	35
Salt mixture†	g	40	40
Liver concentrate‡	g	20	20
Brewer's yeast§	g	10	10
Choline chloride	g	2	2
Inositol	mg	625	625
Thiamin	mg	0.3	0.3
Pyridoxine	mg	0.4	0.4
Calcium pantothenate	mg	0.4	0.4
Manganese chloride	mg	20	20
Vitamin K ("Synkavite")	mg	40	40
Oleum percomorpheum	drops	3	3
Water	ml	250	250

* VioBin Corporation. Contains 3 to 4 mg of α-tocopherol per gram, in addition to unknown quantities of other vitamins.

† P. B. Hawk and B. L. Oser (Science, 1931, 73: 368) with 1 g each of copper sulphate and zinc chloride added to trace elements.

‡ Wilson (20:1). Generously supplied by the pharmaceutical laboratories of Wilson and Co.

§ Purchased from Mead Johnson and Co.

Infective agent.—A type I, A-5 strain pneumococcus, originally isolated from a patient with lobar pneumonia, was obtained through the courtesy of Dr. O. H. Robertson. Its virulence was maintained by monthly rabbit passage. For infection experiments, a subculture was incubated at 37 C for 7 hours in veal infusion broth containing 0.5% dextrose and 1.5 % normal rabbit serum. From this 1.0 ml was transferred to each of several centrifuge bottles containing 150 ml of medium. These bottles were incubated for 4 hours, then centrifuged for 30 minutes at 3000 r.p.m., and the supernatant drawn off with an aspirator. For a preliminary experiment the organisms were resuspended in 100 ml of normal saline. None of the roosters died following infection with this suspension. Therefore, in subsequent experiments, each bottle of centrifuged pneumococci was resuspended in 8.0 to 10.0 ml of saline, depending upon a gross estimation of the turbidity of the culture. The birds were immediately injected via the jugular vein with 3.0 ml of this suspension per kg of body weight. For each experiment the depleted rooster with the greatest percentage weight loss and the comparable control bird were injected with organisms which had been killed by heating to 56 C for 30 minutes. In experiments 1, 2, and 3 direct bacterial counts were not made, in order to keep the time between centrifugation and injection of the

26. Almquist, H. J. 1947, J. Nutrition, **34**: 543–563.

organisms as short as possible. As soon as all birds were injected, however, serial dilutions of the suspension were made and 0.01 ml of several dilutions plated on blood-agar plates. At this time stains were prepared, and these revealed that over 99% of the organisms were gram-positive and encapsulated. For experiments 4 and 5, the organisms were enumerated in a hemocytometer. This was done in experiment 4 as a check on the accuracy of the culture method of counting and to insure the use of a smaller quantity of pneumococci for experiment 5. For experiment 4, the hemocytometer count revealed 6.5×10^{11} diplococci per ml, while the culture showed 3.5×10^{11} per ml.

Mouse protection tests.—Male albino mice of the CFCW strain were obtained from Carworth Farms, New York. The mice used weighed from 16 to 22 g. Comparable groups of three mice each were selected by placing a light (16 to 18 g), medium (19 to 20 g), and heavy mouse (21 to 22 g) in each group. For the tests a serum dilution method was employed, giving one group of mice whole rooster serum, another a 1:2 dilution, and next a 1:4 dilution, etc. A subculture of the pneumococcus was incubated in serum-dextrose broth for 7 hours, and was then counted in a hemocytometer. For the preliminary experiments, 1000 lethal doses were used, but in some of these studies all the mice died. Consequently, in subsequent experiments the infecting dose was reduced to about 10 lethal doses. Serum and culture, 0.5 ml of each, were injected simultaneously from a 1.0 ml tuberculin syringe. Survival of two-thirds of the mice was interpreted as evidence of protection. In each experiment, the count was verified by culture of appropriate serial dilutions, using the method outlined above, and by injecting groups of 3 to 5 mice with one lethal dose (1 to 2 diplococci for this organism) as calculated from the hemocytometric count and with 0.01 and 100 times this dosage. For each experiment the three methods of enumerating the bacteria were in agreement within a factor of 10.

Serum inhibition tests.—In order to determine the effect of normal rooster serum upon the growth of pneumococci in vitro, tubes were prepared with dilutions of sterile serum from normal and protein-depleted roosters in increments of 5%, using veal infusion broth with 0.5% dextrose as the diluent. Control tubes containing saline and broth were also prepared. The total volume used was 2.0 ml. All tubes were inoculated with 0.1 ml of a 6-hour broth culture of pneumococci. They were incubated for 9 hours and the presence or absence of growth noted by gross inspection. The tubes were shaken at frequent intervals, thus minimizing the effect of agglutination upon bacterial growth.[27]

Blood clearance studies.—During each of the experiments 1 to 4, the ability of both groups of birds to clear the circulating blood of pneumococci was studied at some time during the experiment. This was done in either or both of the following ways. Blood cultures were obtained after wiping the leg of the rooster with 70% alcohol, which was allowed to dry. The tibial vein was then punctured with a lancet which had been similarly sterilized and dried. Blood was taken on a 3 mm flamed wire loop and plated on blood agar. After incubation for 24 hours, the number of colonies was counted. Blood smears also were obtained and stained by the McCullough and Dick technique.[28] The number of bacteria per thousand red cells was subsequently determined. In experiment 5 both of these procedures were carried out at 10 minutes, 1, 3, and 7 hours after infection. At 15 minutes, 2, and 6 hours after injection both cultures and smears were obtained in experiment 6.

Temperatures.—These were determined in experiments 1, 2, and 3, at approximately 4-hour intervals for the day preceding as well as the day following infection. For this determination, a rectal thermometer with a maximum reading of 110 F was inserted into the cloaca for two minutes. The temperature readings thus obtained were reproducible and maximal.

Blood protein determinations.—The birds were fasted for 12 hours before bleeding to avoid lipemic serum. They were bled by puncturing the tibial vein with a lancet. The blood was collected in 6 mm tubes, allowed to clot, rimmed, and immediately centrifuged. Serum protein values were determined by the method of Barbour and Hamilton,[29] using a hydrocarbon mixture in which the potassium sulfate standard (specific gravity 1.0267 at 25 C) fell in about 16 seconds. For conversion of specific gravity to protein concentration, the formula of Weech et al[30] was used. The method was checked by micro-kjeldahl determinations on some of the samples and found to agree within ± 0.10 g %.

Blood cell counts.—The literature contains many methods, both direct and indirect, for the enumeration of chicken leucocytes. All of these were tried and were either too cumbersome to be

27. Blake, F. 1917, J. Exper. Med. **26**: 563–580.
28. McCullough, N. B. and Dick, L. A. 1942, Stain Technol. **17**: 153–155.
29. Barbour, H. G. and Hamilton, W. F. 1926, J. Biol. Chem. **69**: 625–640.
30. Weech, A. A., Reeves, E. B. and Goettsch, E. 1936, J. Biol. Chem. **113**: 167–174.

used repeatedly during a short experiment or did not differentiate the thrombocytes from the lymphocytes. Thus it seemed necessary to find a stain which would color the granulocytes quickly without staining the other cells. The granulocytes could then be counted directly, and from the differential count, the total leucocyte concentration could be calculated. To accomplish this, supravital stains were prepared using neutral red, janus green, pyronin, brilliant cresyl blue, crystal violet, methylene blue, cyanin, congo red, methyl green, fluorescein, and nile blue sulphate. With the smears on a warm stage, it was found that nile blue sulphate colored the granules very quickly and did not stain any of the other cells supravitally. Therefore, a modification of Wetmore's technique[31] was used, as follows: The nile blue sulphate was diluted to about 1 part in 50,000 in Locke's solution, giving a clear azure shade. A second solution contained 12% formalin in Locke's. The blood was drawn to the proper level in a certified Trenner Automatic red cell pipette. The dye solution was drawn up until the bulb was about half full. The pipette was agitated gently for 30 seconds to allow supravital staining, after which filling was completed with the formalin solution. Granulocyte counts were made by enumerating all the dark blue cells in the area of the hemocytometer ordinarily used for white cell counts. Both chambers of the hemocytometer were counted, and the result multiplied by 250 to give the number of granulocytes per cmm of blood. Red blood cells were counted in the usual way from the same filling of the counting chamber. Differential smears were stained with Wright's stain and 100 cells were counted by the "battlement" method on each of two slides. From the differential counts, the total white cell count was calculated. White cell counts made in this way on rabbit blood agreed well with the counts obtained using 2% acetic acid as the diluting fluid. Fresh blood must be used, however, as either heparin or the oxalates interfere with the staining of the granules.

Histological studies.—All birds were autopsied within 30 minutes after death, and blood cultures were taken from the heart if the blood was still fluid. Portions of liver, spleen, lung, femoral bone marrow, kidney, heart, pancreas, jejunum, testis, adrenal, and in experiments 1 and 2, skeletal muscle were fixed in Zenker's solution containing 10% formalin. The organs were imbedded in celloidin and sectioned at 6μ. Sections of each organ were stained with hematoxylin and phloxine or with the Lillie modification of the gram stain.[32] For all experiments, duplicate blocks of liver, heart, and kidney were fixed in 10% formalin, sectioned on a freezing microtome, and stained with oil red O.

Titrations.—An attempt was made to demonstrate pneumococcal agglutinins in these birds before infection, using a homologous vaccine and an initial serum dilution of 1:1.[33] Complement was also titrated in normal and protein-depleted serum, using 2 units of commercial amboceptor, 0.5 ml of sensitized cells, and the appropriate dilution of rooster serum in a total volume of 2.0 ml. The tubes were incubated in a water bath at 37 C for 2 hours, then placed in a refrigerator overnight and read the following morning.

TABLE 2.—*The effect of protein-depletion upon weight, serum protein, diet consumption, and survival of infection with type I pneumococcus.*

Exp. no.	Number of birds	Initial data: Wt. in g	Initial data: Serum protein-grams percent	Depletion period: Diet	Depletion period: Duration in days	Depletion period: Diet consumed cal./M²	Time of infection: Wt. in g	Time of infection: Serum protein-grams percent	Dosage of pneumococci per kg body weight	Percent surviving
1	8	738	3.86	D	70	1500	447	2.26	2.3×10^{12}	12.5
1	8	682	3.68	C	70	2400	1316	3.99	2.3×10^{12}	67.5
2	5	759	4.07	D	110	2600	430	2.40	9.0×10^{10}	0
2	5	760	3.71	C	110	2200	1544	3.79	9.0×10^{10}	60
3	9	737		D	120	2100	443	1.97	5.4×10^{11}	0
3	9	738		C	120	1800	1669	4.02	5.4×10^{11}	33
4	7	744		D	132		513		1.5×10^{12}	0
4	6	741		C	132		1679		1.5×10^{12}	50
5	5	752		D	121		446		7.5×10^{8}	80*
5	5	732		C	121		1814		7.5×10^{8}	100*
6	8	727		D	129		447		5.0×10^{11}	†
6	9	725		C	129		1808		5.0×10^{11}	†

* These birds received a dosage of pneumococci which was calculated to be nonlethal. Despite this, one of the protein-depleted birds died.
† Sacrificed at intervals.

31. Wetmore, P. 1940, Science, **92**: 386.

32. Lillie, R. D. 1928, Arch. Path. **5**: 828–838.
33. Wissler, R. W. 1947, J. Infect. Dis. **80**: 250–263.

EXPERIMENTAL RESULTS

The results of the mortality experiments are given in table 2. These demonstrate that a dosage of pneumococci which was fatal to half of the control birds resulted in the death of all but one of the group of 29 protein-depleted roosters. Statistically, the differences in mortality in each of these experiments is of borderline significance. However, the sum of these experiments yields a highly significant difference in mortality. In none of these experiments did the birds injected with heat-killed organisms succumb. Indeed, death does not follow the use of 4 to 5 times the usual dosage of heat-killed organisms. Such animals, however, have a temperature and leucocyte response similar to that observed when live organisms are injected.

The degree of protein depletion obtained in these experiments is also shown in table 2. There it may be observed that the birds fed diet D lost from 31% (exp. 4) to 43% (exp. 2) of their original weight, whereas those given diet C approximately doubled their weight. Likewise, the depleted birds lost about 41% of their serum protein concentrations, but the controls maintained their original levels. Thus the roosters given diet D showed severe protein deficiency.

Was general starvation produced or only protein restriction? Although it is true that the birds given diet D ate progressively less diet, it must be remembered that they were also losing weight. Further, it has been shown that energy requirements are directly proportional to surface area.[34] Thus, when diet intake is calculated in terms of grams eaten per square meter of surface area, using Mitchell's formula,[35] it is apparent that for experiment 1 the depleting birds ate almost as well, and in experiments 2 and 3 ate much more than did the control animals (table 2). There are two reports in the literature of the energy requirements of cockerels. One report, based on two groups of birds weighing about 2 kg, concludes that 1500 to 1700 cal./M^2/day are required.[36] Another more extensive study, reports that for birds of the weight of the depleted animals, about 1350 cal./M^2/day are required. Of this amount, about 1100 calories are used for maintenance, activity, etc., the remainder supplying energy required for growth. Birds weighing 1300 to 1800 g were reported to require about 1200 cal./M^2/day for maintenance and growth.[37] Thus the birds used in these experiments suffered no caloric restriction. Since vitamins, particularly those of the B-complex, take part in metabolic interchanges, and the ratio of vitamins to carbohydrate, fat, and minerals remained constant in these diets, the vitamin intakes were sufficient for the intermediary metabolism of these substances. Further, none of the known signs of vitamin deficiency[38] were ever seen, even in the most depleted roosters. In short, animals fed diet D represent as nearly as possible a pure protein deficiency, complicated only to the extent that proteins are needed internally as enzymes and hormones which are necessary for carboyhdrate and fat metabolism. The importance of this complication should not be underestimated, however.[39]

The remainder of this study was con-

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35. Mitchell, H. H. 1930, J. Nutrition, **2**: 443–449.
36. Titus, H. W. 1929, Poultry Sci. **8**: 80–84.
37. Mitchell, H. H., Card, L. E. and Hamilton, T. S. 1930, Proc. 4th World's Poultry Congr. 323–328.
38. Lee, C. D. 1942, North Am. Vet. **23**: 394–400.
39. Wissler, R. W., Benditt, E. P., Steffee, C. H. and Woolridge, R. L. 1949, Federation Proc. **8**: 375.

TABLE 3.—*The influence of protein-depletion upon mouse-protecting substances in rooster serum (exp. 2).*

Serum dilution protecting mice*	Rooster data		
	Number of serums pooled	Diet	Outcome of subsequent pneumococcal infection in roosters
None†	3	D	All died
1:2	2	D	Both died
1:2	3	C	⅔ survived
1:4	3	C	⅔ survived

* All mice given 1000 lethal doses of pneumococci.
† Whole serum failed to protect mice against this dosage of organisms.

cerned with trying to elicit the mechanism by which loss of dietary protein resulted in loss of natural resistance. The differences in the various components studied thereby give some data on the nature of natural resistance per se.

The first facet of natural resistance investigated was the in vivo protective power of the serum, using the mouse protection test and over 700 mice. As an example, the results obtained in experiment 2 are shown in table 3. In 10 experiments in which serums from protein-depleted and normal birds were compared, the depleted serums showed less protective action than the controls 9 times. In only one instance did the control serum show the same protection as that from the corresponding depleted birds, and depleted serum was never found to have more protective action than control serum. There are some internal discrepancies in the comparison of the data from experiment to experiment, possibly due either to a "zone phenomenon" in some of the lower dilutions of culture, as reported by Goodner,[40] or to differences in the length of time the rooster serums were stored before the mouse-protection studies were done. Some of the protective action is removed by Seitz filtration, and for this reason the serums were handled with sterile precautions. However, the results indicate clearly that normal roosters which have never been injected with pneumococci have circulating substances which can protect mice against pneumococci, and that these substances are decreased when the rooster is deprived of protein for long periods.

In addition to the mouse-protection studies, the antipneumococcal effects of serum from normal and protein-depleted roosters were tested in vitro. Two experiments were performed with similar results. Table 4 presents the findings in the second of these experiments, after incubation of the pneumococci in various dilutions of serum for 9 hours. Here it may be seen that for either total or partial inhibition of pneumococcal growth, lower concentrations of control serum than of serum from depleted birds were necessary. Doubling the time of incubation resulted in slight to moderate growth with 50% serum from control birds as contrasted to luxuriant growth in tubes containing 60% serum from depleted birds. This again demonstrates the presence of antipneumococcal substances in the serum of normal roosters, and shows also that a prolonged period of protein inanition decreases the effectiveness of these substances. It should be pointed out that these anti-

TABLE 4.—*The effect of serum from normal and protein-depleted roosters upon the growth of pneumococci in vitro (9 hours).*

Source of serum	Lowest concentration of serum showing complete inhibition* (percent)	Lowest concentration of serum showing partial inhibition* (percent)
Depleted roosters		
Sample 1	60	30
Sample 2	45	40
Normal roosters		
Sample 3	40	30
Sample 4	30	25

* Determined by comparison with control tubes in which saline instead of rooster serum was diluted with dextrose-broth.

40. Goodner, K., Horsfall, F. L. and Miller, D. K. 1935, J. Exper. Med. **62**: 359–407.

pneumococcal properties were evaluated in cell-free media, thus avoiding the influence of any phagoycytosis-promoting principles.

The effectiveness of these antipneumococcal substances when aided by phagocytosis was evaluated by studying the ability of the animal to clear the blood of injected pneumococci. During all 6 experiments, blood cultures and/or the ratio of pneumococci to red cells were examined during the course of the experiment. During experiments 5 and 6, repeated cultures and smears were studied at from 10 minutes to 7 hours after infection. These experiments demonstrated at each bleeding that if the dosage of pneumococci was high (10^{11} organisms per kg or greater), the control birds could significantly reduce the number of pneumococci more rapidly than the depleted bird. This was true in the control birds which succumbed to the infection as well as in the survivors. Further, if, as in experiment 5, a sublethal dosage of pneumococci was employed (10^9 organisms per kg), the control birds cleared their circulation of pneumococci about 4 hours sooner than did the depleted birds. The computation of the number of pneumococci per ml of blood, obtained by multiplying the pneumococcal red cell ratio by the erythrocyte count, does not alter these conclusions.

In order to assess the phagocytic activity of these roosters in experiment 6, paired control and protein-depleted birds were sacrificed at random at ¼, ½, 1, 2, 3, 4, 5, and 7 hours after infection, an additional control bird being sacrificed also at 10 hours. The depleted animal remaining to be sacrificed at 7 hours expired 6½ hours after infection. In birds, it has been shown that the liver is the major phagocytic organ,[3,4] and for that reason it was studied more intensively. The number of diplococci was counted in 50 consecutive oil immersion fields (magnification 1225×), taking care to avoid large blood vessels which sometimes contained many pneumococci. Differentiation was made between intra- and extracellular organisms. The averaged results for some of the birds are presented in table 5. Representative sections are shown in plate I. It should be noted that the liver of the control bird seemed to concentrate the circulating organisms. As evidence for this, the difference in the number of free pneumococci in the livers of control and depleted birds killed 15 minutes after infection is statistically significant, although both birds received the same dosage of organisms per kg of body weight. This is in agreement with the observation reported above, that the control birds clear their circulating blood of pneumococci more rapidly than do the depleted roosters. In the control birds, phagocytosed pneumococci were present 15 minutes after injection, whereas intracellular organisms were not seen in the depleted birds before one hour after infection. Further, in the depleted bird, it is not until 5 hours have elapsed that the intracel-

TABLE 5.—*Number of pneumococci per oil immersion field in the livers of protein-depleted and control roosters sacrificed at intervals after infection (exp. 6).*

Diet	¼ hr.		1 hr.		3 hr.		5 hr.		7 hr.	
	Free	Phago-cytosed	Free	Phago-cytosed	Free	Phago-cytosed	Free	Phago-cytosed	Free	Phago-cytosed
D	2.7	0	4.5	1.3	4.4	4.0	5.7	21.9	170*	30*
C	4.0	1.0	4.0	7.3	1.0	14.4	1.6	46.2	0.4	11.7

* This bird died 6½ hours after injection. All others were sacrificed at the times indicated.

lular pneumococci exceed the extracellular ones, although this is true at one hour in the control. In the liver of the control bird sacrificed at 10 hours, a total of 3 extracellular pneumococci was seen in the 50 fields examined. There were 10.6 organisms per field in Kupffer cells. It is apparent, therefore, that in the protein-depleted birds the pneumococci are neither as quickly nor as effectively phagocytosed. This results in the persistence of circulating organisms as noted in the preceding paragraph.

Examination of the sections of other organs obtained in experiment 6 revealed the following changes. In the spleen, the pneumococci tended to accumulate in tiny areas of the red pulp to form small abscess-like lesions. These had an acidophilic background and usually contained abundant polymorphonuclear leucocytes. The cellular density of this organ makes numerical evaluation of phagocytosis more difficult, but even casual study of these sections was sufficient to demonstrate that phagocytosis was much more active in the controls than in the depleted birds. Further, there was no phagocytosis noted in any of the sections of bone marrow of protein-depleted birds, whereas this was found, though not prominent, in the marrow of control birds. In the depleted bird there was marked atrophy of the marrow, which was never over 10% cellular, and showed predominantly erythropoietic activity with little or no granulocytopoiesis. In the controls, the marrow was 50 to 90% cellular, with granulocytopoiesis predominating. There were occasional small lymphocytic nodules. The depleted birds showed a general lymphocyte depletion, with fewer lymphocytes than normal in the spleen, and none in the marrow, intestine, or liver, which are normal sites of lymphocyte production in the bird.[41] In the depleted birds, there was an accumulation of hemosiderin in the liver cord cells as well as in Kupffer cells. In the lung, kidney, heart, adrenal, intestine, pancreas, and testis, pneumococci were found only within blood vessels, and there was no phagocytosis. The lungs, kidneys, and adrenals of the depleted birds appeared essentially normal. There was moderate atrophy of the heart, intestinal mucosa, and pancreas in the depleted birds, and marked atrophy of the testis, with complete absence of spermatozoa. Study of the sections of normal and depleted birds dying of pneumococcal infection in experiments 1 to 4 revealed a similar cytological picture. They differed from the above description only in that the blood vessels contained huge numbers of pneumococci, as phagocytosis had obviously not kept pace with the growth of the organism. In all of these experiments sections of heart, liver, and kidney which were stained for fat revealed occasional foci of fine droplet fat in only a few of the animals. The frequency of the fatty degeneration was the same in

41. Jordan, H. E. 1936, Am. J. Anat. **59**: 249–297.

FIG. 1.—The liver of a control bird sacrificed ½ hour after infection. Note the Kupffer cell near the center containing a phagocytosed pneumococcus. × 2500.

FIG. 2.—The liver of a control bird killed 5 hours after infection. There are numerous clumps of phagocytosed pneumococci and occasional extra cellular organisms. Such clumping was not observed among the depleted birds. × 2500.

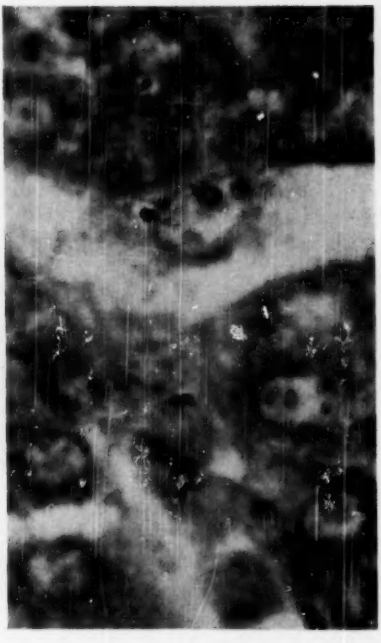
FIG. 3.—The liver of a depleted bird dying 6½ hours after infection. Most of the pneumococci are extracellular. × 2500.

FIG. 4.—The liver of a control bird sacrificed 7 hours after infection. Note the few remaining intracellular organisms. × 2500.

1

2

3



4

PLATE I

TABLE 6.—*Body temperatures before and during infection of protein-depleted and control roosters (exp. 2).*

Animal no.	1½ hours pre-infection (F)	Postinfection temperatures (F)			Survival time (hours*)
		1½ hours	6 hours	11 hours	
		Low protein diet group			
30	104.2	106.4	97.8		6½
31	104.2	100.5			6
32	103.4	98.7	101.1		6¾
33	104.6	106.8	105.0		8
34	104.2	106.2			6
		Control diet group			
35	106.5	105.2	106.2	108.6	S
36	106.8	107.4	107.6	107.0	S
37	107.4	108.3	108.4	108.7	14¼
38	107.6	107.4	108.2	107.8	13½
39	107.0	108.2	107.0	109.2	S

* S = survived.

the protein-depleted birds and in the controls.

Studies of the body temperature of 50 birds gave no evidence that it contributes to natural resistance. The results obtained in experiment 2 are given in table 6. Although it is true, in general, that the protein-depleted birds had lower body temperatures than did the controls, the one depleted bird to survive infection also had a low body temperature at the time of infection (104.5 F). Further, among the control birds the outcome of the infection could not be predicted from the temperature readings before injection. Although the temperatures of the depleted birds had a tendency to drop after infection, they rose in some cases to 108 F and above, but the birds died nevertheless. In addition, it was possible to obtain excellent growth of this strain of pneumococcus when it was incubated at 108 F (42 C) and good, though not luxuriant, growth with incubation at 109 F. Thus no evidence has been obtained to support the theory that natural resistance to pneumococcus is primarily a function of body temperature in the bird.

Leucocyte counts were done in experiments 1, 2, and 3 before infection and at about 4 and 12 hours after infection. As an example, the results obtained in experiment 1 are presented in

TABLE 7.—*The influence of a low protein diet and subsequent infection upon the erythrocytes, leucocytes, and the differential counts (exp. 1).*

Animal no.	Preinfection counts				Counts 4 hours after infection			
	RBC, million/cmm	WBC, 1000/cmm	Granulocytes, 1000/cmm	Non-granulocytes, 1000/cmm	RBC, million cmm	WBC, 1000/cmm	Granulocytes, 1000/cmm	Non-granulocytes, 1000/cmm
				Low protein diet group				
1	2.9	18.42	5.25	13.17	2.5	5.35	4.25	1.10
2	3.0	15.96	5.25	10.71	2.2	13.71	12.00	1.71
3	2.4	14.91	4.25	10.66	2.7	16.67	15.00	1.67
4	1.9	5.38	2.50	2.55	1.3	4.10	2.70	1.40
5	2.6	8.54	5.25	3.29	2.6	6.44	4.25	2.19
6	2.1	8.49	2.25	6.24	1.7	7.40	6.25	1.15
7*	2.4	8.52	3.75	4.47	2.2	15.43	13.50	1.93
8	2.2	15.32	4.75	10.57	2.1	6.83	6.25	0.50
Average	2.4	11.95	4.15	7.80	2.1	9.50	8.05	1.45
				Control diet group				
11*	3.3	15.00	5.25	9.75	3.2	15.51	12.25	3.26
12	2.9	30.95	3.25	27.70	2.7	42.55	30.00	12.50
13*	2.9	12.26	3.25	9.01	2.7	27.74	22.75	5.01
14*	3.5	18.48	4.25	14.23	2.7	15.23	11.50	3.73
15	3.0	9.14	4.25	4.91	2.9	44.24	30.75	13.49
16	2.8	22.73	5.00	17.73	2.7	57.06	46.50	10.56
17*	2.5	6.41	1.25	5.16	2.3	5.40	3.75	1.65
18*	2.6	40.22	26.75	13.47	2.0	28.40	23.00	5.40
Average	2.9	19.40	6.65	12.75	2.7	29.50	22.55	6.95

* Survived the infection.

table 7. In general, both control and depleted birds showed granulocytosis, more marked in the controls, during the infection. The most difficult feature to explain, however, is the lymphocytopenia, which was present in all experiments and was greater in the protein-depleted birds. Among the control roosters, neither the rise of granulocytes nor the fall in lymphocytes correlated with survival of the infection. The red blood cell counts suggested a slight anemia with the progression of the infection, but the differences were just outside the expected variation in serial erythrocyte counts.

Titration of the serums before pneumococcal infection did not reveal pneumococcal agglutinins, in either normal or protein-depleted birds. Titration of the complementary activity of the serums of 9 protein-depleted and 6 control birds demonstrated a tendency for greater activity in the serums of the depleted birds. For the latter an average of 0.12 ml of serum, and for the controls an average of 0.20 ml of serum was necessary to lyse cells in the presence of 2 units of amboceptor. Thus the differences in the resistance of normal and protein-depleted roosters to pneumococcal infection cannot be ascribed to either natural agglutinins or to a loss of complement in the depleted birds.

DISCUSSION

Several previous investigators have studied the role of body temperature as the primary mechanism of natural resistence. Pasteur first reported that chilling reduced the immunity of pigeons to anthrax infection. Subsequently, it was shown that lowering body temperature to 40 C (102 F) or below by the use of pyramidon[42] or by deprivation of vitamin B[43,44] would increase the susceptibility of pigeons to pneumococcal infection. However, this is not a function of temperature per se since the organism can be cultured easily at temperatures above this "critical" level. Further, the phagocytic mechanism is less effective under such conditions. Rabbits which had been chilled showed an increased mortality following infection with avirulent pneumococci, due, presumably, to a diminished inflammatory response.[45] Similarly, alcoholized, immunized rabbits become hypothermic and lose their resistance to intradermal pneumococcal infection because of their failure to produce a localizing lesion. Simple hypothermia has no effect on susceptibility.[46] Further, when the anterior chamber of the eye of pigeons was infected with *B. pyocyaneus*, it was noted that the control animals had more leucocytes and fewer extracellular organisms than did those birds previously chilled by immersion in ice water.[47] In addition to these reports in the literature, the experiments reported here offer no evidence that body temperature plays any direct role in the outcome of pneumococcal infection in a naturally resistant animal.

There are many divergent reports on the mouse-protecting power of serums of naturally resistant animals. Cole and Smirnov have reported that pigeon serum increases the susceptibility of mice to pneumococcal infection.[48] From the data given, it is difficult to explain their results, except to point out that

42. Strouse, S. 1909, J. Exper. Med. **11**: 743–761.
43. Findlay, G. M. 1922, Lancet, **1**: 714–715.
44. Findlay, G. M. 1923, J. Path. & Bact. **26**: 485–495.
45. Meschenheim, C., Duerschner, D. R., Hardy, J. M. and Stoll, A. M. 1943, J. Infect. Dis. **72**: 187–196.
46. Pickrell, K. L. 1938, Bull. Johns Hopkins Hosp. **63**: 238–260.
47. Wagner, K. E. 1890, Ann. Inst. Pasteur, **4**: 570–602.
48. Cole, R. I. and Smirnov, M. R. 1908, Bull. Johns Hopkins Hosp. **19**: 249–252.

their experiments employed a total of 24 mice, and that relatively large volumes (up to 2.25 ml) were injected into some of them. Kyes stated that serum from normal roosters did not protect mice but gave no details as to the handling of the serum.[49] Kelley also recorded the lack of protecting substances in normal rooster serum.[50] He used serum which was filtered through a Berkfield candle and stored at 5 C. The length of time the serum was stored was not given. In the experiments reported here, there was a tendency for the protective action to disappear when the serum was stored in a freezer for 56 days. Filtration also reduced the mouse-protective titer of the serum. Perhaps a combination of these factors could explain the results obtained by Kyes and by Kelley. The pneumococcus has been reported as a cause of abortion in sows, but the organism was not positively identified.[51] In other studies it has been demonstrated that swine serum protects mice against 19 types of pneumococci[52] and that chicken serum protects at least against types I and II.[53] The latter investigators also showed that the protective substances were in the globulin fraction, and that they were type-specific. This specificity poses the question as to whether these substances are natural or acquired bodies, as it seems strange to postulate that the normal chicken should contain humoral substances which will protect specifically against each type of pneumococcus. However, it would be equally difficult to postulate that the birds or the swine had been infected with each of the types of pneumococci and had developed antibodies against them. The fact that the experiments reported here were carried on over a period of three years, during which time only four animal caretakers had contact with the birds, would argue against exposure of all the birds to pneumococcal infection from human carriers, in view of the low carrier rate for type I pneumococci.

The demonstration of bacteriostatic substances in rooster serum substantiates a previous report that pneumococci will grow in pigeon serum but with a longer latent period.[54] With small numbers of bacteria, chicken serum has been shown to be bactericidal.[55] Further, other experiments testing the bactericidal action of serum of rats deficient in vitamins A, B, or D, have shown reduction in potency in serum from rats with severe deficiency of these vitamins.[56] Unfortunately, since diet consumption was not reported and inanition controls were not included, it is only possible to suggest that if these rats had the usual anorexia associated with vitamin deficiency, they were probably also protein-deficient. Therefore, the data in the literature, both with regard to mouse protective action and bacteriostatic or bactericidal action, seem to indicate the probability that these factors are present in the serum of normal birds. This is borne out by the experiments reported here, and in addition, it has been shown that protein deficiency depresses such activity.

The role of phagocytosis has been emphasized by many investigators. In the dog, it has been found that pneumococci disappear rapidly from the blood

49. Kyes, P. 1911, J. A.M.A. **56**: 1878–1881.
50. Kelley, W. H. 1932, J. Exper. Med. **55**: 877–888.
51. Stitz, B. 1941, Rev. Med. Vet. **23**: 250–253.
52. Victoroff, L. K. and Klotz, L. I. 1938, Rev. d'immunol. **4**: 175–196.
53. Bull, C. G. and McKee, C. M. 1921, Am. J. Hyg. **1**: 284–300.
54. Bull, C. G. and Bartual, L. 1920, J. Exper. Med. **31**: 233–251.
55. Heist, G. D., Solis-Cohen, S. and Solis-Cohen M. 1918, J. Immunol. **3**: 261–276.
56. Findlay, G. M. and MacLean, I. 1925, Biochem. J. **19**: 63–70.

stream and are phagocytosed in the liver, spleen, and lung.[5] Further, in rabbits, it has been shown that although the serum contains antibodies, leucocytes are necessary to overcome an intradermal pneumococcal infection.[46,57,58] Indeed, avirulent pneumococci may be fatal to rabbits which are leucopenic.[59] The relationship of phagocytosis to these experiments is emphasized further by the reports that protein depletion of rats results in reduction of the phagocytic activity of the leucocytes[24,25] and macrophages.[24] Moreover, the leucocytic response of a protein-depleted animal is quickly exhausted.[60] These findings, which are confirmed in the present study, may be explained in two ways: (1) The reduction in protective activity of the serum may decrease the opsonization of pneumococci, and thus fewer of them are phagocytosed. Unfortunately, sufficient serum was not obtained during the course of these experiments to concentrate the protective fraction in order to determine the resistance of protein-depleted birds which had been passively protected with this concentrate. (2) The protein-starved animal has all its metabolic processes slowed, and this slowing is reflected in the lowering of body temperature. Therefore, the phagocytic cells of such starved birds may be unable to digest their engulfed particles as well as do those of normal birds. This digestion may be further inhibited by the presence of large amounts of hemosiderin in the Kupffer cells of the depleted birds. Chemically, of course, a lowering of temperature will decrease the speed of reactions, and this factor may influence the phagocytic defense of the protein-depleted bird. Further, the phagocytic cells, metabolically depressed, may be less able to ingest foreign material with which they have contact. I have no evidence to determine whether either or both of these factors is responsible, but the decreased phagocytic response of the protein-depleted animal is definite.

In this study, as in those of previous workers, the exact mechanism responsible for death is not evident. However, most of the birds dying of pneumococcal infection became cyanotic, and many of them showed petechial hemorrhages in the legs and in the pericardium. Occasional birds which were bled as they were dying have also shown marked hemolysis. Toxins have been demonstrated by several authors,[61,62,63] and perhaps the "purpura producing substance"[64,65] was responsible for the petechiae noted. At any rate, the absence of fatty changes in the liver and heart suggests that, if toxins are important, the duration of illness in these birds was too short to allow the usual morphological changes associated with toxemia to appear. Further evidence against a toxemic death is the fact that severely protein-deficient birds injected with large quantities of vaccine never succumbed. Thus the precise mechanism of death remains obscure.

SUMMARY AND CONCLUSIONS

Forty-two young roosters were depleted of their protein stores by pro-

57. Rich, A. R. and McKee, C. M. 1934, Bull. Johns Hopkins Hosp. **54**: 277–311.
58. Lushbaugh, C. C. 1943, J. Immunol. **46**: 151–159.
59. Rich, A. R. and McKee, C. M. 1939, Bull. Johns Hopkins Hosp. **64**: 434–446.
60. Asirvadham, M. 1948, J. Infect. Dis. **83**: 87–100.
61. Coca, A. F. 1936, J. Immunol. **30**: 1–31.
62. Dick, G. F. and Boor, A. K. 1938, Arch. Path. **26**: 93–95.
63. Carpenter, C. M., Barbour, G. M. and Ackerman, H. 1940, J. Bact. **39**: 27–28.
64. Reimann, H. A. and Julianelle, L. A. 1926, J. Exper. Med. **43**: 87–106.
65. Julianelle, L. A. and Reimann, H. A. 1927, J. Exper. Med. **45**: 609–618.

longed feeding of a diet almost free of protein. The resistance of these animals, in comparison with that of birds fed an adequate diet, to pneumococcal infection was investigated. The mortality, phagocytic, temperature, and leucocytic responses to infection were studied. The serums were tested for antipneumococcal substances in vivo and in vitro. Agglutinin and complement titers were determined before infection. The conclusions are as follows:

1. Only one of the group of 29 protein-depleted birds survived an infecting dose of pneumococci which was fatal to half of the normal birds.

2. Serums of protein-depleted birds show less mouse-protective action and are less potent bacteriostatic agents than are serums from normal birds.

3. Phagocytosis is depressed in the depleted birds as evidenced by their inability to clear the circulating blood of pneumococci, as well as the observation of retardation and ineffectiveness of phagocytosis by the Kupffer cells during the infection.

4 Although the depleted bird is usually hypothermic, a normal body temperature does not prevent fatality following injection of pneumococci. Further, among the control birds, survival can not be predicted from either the preinjection or postinjection temperatures.

5. Despite differences observed, the leucocyte response of both normal and depleted birds offers no clue as to the outcome of the infection or the fundamental mechanism of natural resistance.

6. "Natural" agglutinins were not demonstrated.

7. The complement titer does not decrease in the protein-depleted rooster.

MULTIPLICATION OF THE AGENT OF FELINE PNEUMONITIS IN THE YOLK SACS OF DEAD CHICK EMBRYOS

EMILIO WEISS

From the Department of Bacteriology and Parasitology, The University of Chicago, Chicago 37, Illinois

Rickettsia prowazeki was recently grown in the yolk sac of dead chick embryos by Rabinowitz et al (1948). Growth was attributed to the prolonged survival of cells of the extraembryonic membranes in dead embryos. They showed that embryos killed by chilling on the third day of their development and maintained at 37 C for as long as 16 days still contained cells which grew out in plasma clots. These cells were believed to have a low metabolism and, therefore, in accordance with the observations of Zinsser and Schoenbach (1937), to be especially well suited for the development of rickettsiae. In fact, the concentration of rickettsiae per cell was possibly higher in the dead than in the living embryos. Because of the similarity between rickettsiae and the agents of the psittocosis-lymphogranuloma group (Rake, 1948), it seemed of interest to grow a typical member of this group, the agent of feline pneumonitis, in dead chick embryos.

While this study was in progress, Lahelle and Horsfall (1948) reported the multiplication of influenza virus in the allantoic sac of dead chick embryos to an extent comparable to its multiplication in living ones, although a longer incubation period was required. These results intensified interest in dead chick embryos as tissue culture mediums for rickettsial and viral agents.

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The technical assistance of Miss Margaret K. Itatani is gratefully acknowledged.

MATERIALS AND METHODS

The strain of feline pneumonitis virus was the same as that used previously (Weiss, 1949). A pool of organisms was prepared by shaking heavily infected yolk sacs with glass beads in a mechanical shaker for one-half hour in the presence of enough veal infusion broth to make a 10% emulsion. The resulting emulsion was dispensed into several tubes and stored in the dry ice box at −76 C.

Embryos of white Leghorn eggs were used. Dead embryos were obtained by incubating the eggs for 5 to 6 days at 37 C and in early experiments by chilling them at 5 C for 20 to 24 hours. As several embryos survived such treatment, the eggs were chilled for 40 to 44 hours in later experiments. After the eggs were reincubated for 6 to 8 hours, the embryos were examined for cessation of spontaneous movement and vascular pulsation, and, when harvested later, for their failure to develop beyond the 6th day. Embryos did not survive the more prolonged chilling period.

To determine the presence of viable virus, to estimate LD_{50} end points, and for serum neutralization tests, live 6- to 8-day-old chick embryos were used. LD_{50} end points were determined 10 days after inoculation by the method of Reed and Muench (1938), using 5 10-fold dilutions and 10 eggs for each dilution. In serial passage experiments the titer of the virus was roughly estimated by the single dilution method described by Golub (1948). All dilutions were made in veal infusion broth. The desired amount of virus suspended in 0.25 ml was injected into a yolk sac with a 22 gauge needle $1\frac{3}{4}$ inches long.

All dead embryos and large representative numbers of living embryos were routinely tested for bacterial contamination.

Elementary bodies were demonstrated on Macchiavello-(Zinsser et al, 1939) and Gram-stained smears.* For histological examinations, unwashed yolk sacs were fixed in Zenker-formol, embedded in celloidin, sectioned and stained with hematoxylin-eosin-azure II.

* When the Gram stain is employed, the agent of feline pneumonitis as well as the agent of murine pneumonitis retains the gentian violet and stands out clearly provided the slides are only lightly decolorized.

Serum neutralization tests were carried out in chick embryos with a rooster immune serum prepared by Miss St. John. The procedure was similar to that of the test in mice described by St. John and Gordon (1947).

RESULTS

Serial passage of the agent in dead embryos.—The agent of feline pneumonitis was serially passed in dead embryos 20 times. A first group of 4 dead embryos

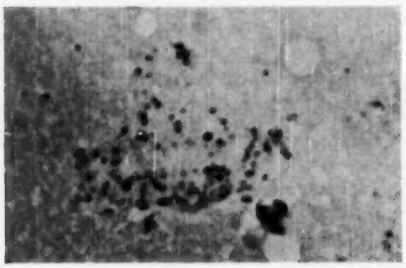

FIG. 1.—Elementary bodies of the virus of feline pneumonitis. The virus was harvested on the 7th day after inoculation into the 18th successive dead embryo (impression smear, Gram stain).

was each inoculated with a heavy inoculum (10^7 LD_{50}) and the yolk sacs harvested after 4 days incubation at 37 C. The high concentration of virus and time of harvesting were chosen arbitrarily to detect a minimal amount of growth. They were not expected to yield highly infected sacs. A pool prepared from the harvested embryos contained a lower concentration of virus than the original inoculum, and the titer continued to drop through the 4th passage. Approximately 10^5 LD_{50} were obtained from each yolk sac of the 4th passage. From then on the titer remained constant.

In a second short series only 10^5 LD_{50} were used for the first inoculum, but the yolk sacs were harvested again on the 4th day. The titer of the virus remained constant throughout the first 5 passages.

Microscopic features.—Elementary bodies were demonstrated in Macchiavello- and Gram-stained smears prepared from yolk sacs of most of the dead embryos (fig. 1). They were, however, somewhat fewer in number than in the sacs of living embryos, even though living embryos were inoculated with virus passed several times through dead embryos.

Sections were prepared from 4 dead embryos of the 8th passage, 2 of the 18th passage and 4 living embryos inoculated with the 7th passage. Vesicles indistinguishable from those described previously (Weiss, 1949) were found in all sections. However, fewer vesicles were seen in the dead embryos than in the living ones. No obvious difference was noted in the appearance of the yolk cells, which, presumably, were alive whether the embryos were alive or dead.

Virulence for chick embryos and for mice.—The agent of feline pneumonitis is highly virulent for chick embryos. As Golub (1948) pointed out for other agents of the group, a characteristic slope is obtained when the concentration of the inoculated virus is plotted against the average day of death of the embryos (ADD). For example, the slope of feline pneumonitis virus differs markedly from that of the less virulent murine pneumonitis virus (fig. 2). The former slope has a difference in ADD between two consecutive dilutions of virus of approximately half a day, while the latter has a difference of more than one day.

The virulence of the strain of feline pneumonitis passed in dead embryos 17 times (D×17) was determined. A concentrated pool of D×17 was prepared by harvesting 10 dead embryos 6 days after inoculation. As shown in figure 2, its titration slope closely resembled the original strain of feline pneumonitis. The conclusion may therefore be reached that the virulence of the virus

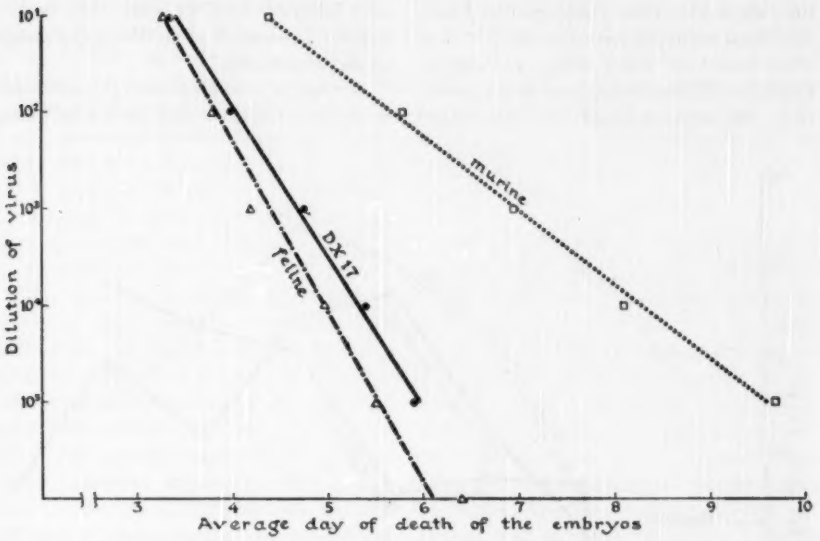

FIG. 2.—Relative virulence of the agents of feline pneumonitis before (feline) and after 17 passages through dead embryos (DX17) and of murine pneumonitis for the chick embryo.

for living embryos remained unaltered after 17 passages in dead embryos.

Although the original strain of feline pneumonitis had lost most of its virulence for mice because of numerous passages in eggs, it still produced typical pulmonary lesions when instilled intranasally in high concentrations into mice. Numerous elementary bodies were found in impression smears from consolidated portions of the lungs. The DX17 strain behaved similarly.

Serum neutralization test.—In an attempt to find a simple method of identifying the virus, a serum neutralization test was carried out in eggs. The results are shown in table 1. The difference in ADD of 2.2 days between protected and unprotected embryos inoculated with the original strain corresponded to a difference of at least three consecutive 10-fold dilutions of virus (fig. 2). In view of the work of Hilleman (1945) and St. John and Gordon (1947) in mice, this test seemed reliable for identifying the virus. The ADD was also lengthened 2.5 days with the same serum and DX17 virus. Hence, the virus had not changed antigenically during its passage in dead embryos.

Rates of growth in living and dead embryos.—The rate of growth of the orig-

TABLE 1.—*Serum neutralization test in chick embryos of the virus of feline pneumonitis (original) and after 17 passages through dead chick embryos (DX17).*

Strain of virus	Number of eggs used	Dilution of virus	Type of serum	Average day of embryo death	Protection in days
Original	10	10^{-4}	Normal	5.2	
Original	10	10^{-4}	Immune	7.4	2.2
DX17	10	10^{-3}	Normal	4.9	
DX17	10	10^{-3}	Immune	7.4	2.5

inal virus was then compared in living and dead embryos with that of D×17 in dead embryos. Each strain was inoculated into 27 dead embryos, and, in addition, the original strain was inoculated into 24 living embryos. The inoculum in every case was approximately $10^{5.2}$ LD_{50} per embryo as shown in figure 3. Groups of three yolks sacs from each group were harvested immediately after inoculation and at daily intervals for 7 or 8 days. The LD_{50} per gram of yolk sac of each group was then determined. The results are shown in figure 3. The D×17 strain was labelled D18, because this experiment represented an additional passage in dead embryos.

A typical growth curve was obtained with the original strain grown in living embryos. The titer of virus collected from the yolk sacs immediately after inoculation was low, $10^{2.7}$. Had the virus been evenly distributed throughout the egg, a titer of $10^{3.5}$ per gram of yolk sac would have been expected. A one day lag period, during which no growth was demonstrated, was followed by two days of logarithmic growth. The

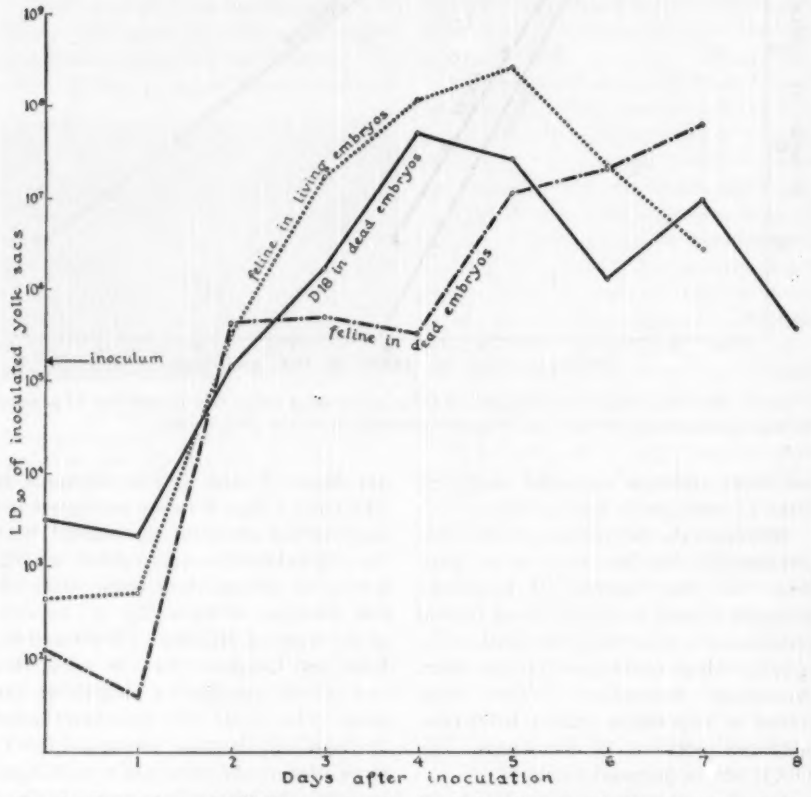

FIG. 3.—Rates of growth of feline pneumonitis virus before (feline) and after 18 passages in dead embryos (D18) in the yolk sacs of living or dead embryos.

rate of growth tapered off between the 3rd and the 5th day. It was interesting to note that although the virus killed the embryos in 4 days, the peak of viral growth was reached at the 5th day, i.e., a day after the death of the embryos. After the 5th day the titer dropped fairly rapidly. At the peak of growth each gram of yolk sac contained approximately 1000 times the total amount of virus injected into the embryo.

The growth curve of the original strain in dead embryos revealed some inconsistencies which were possibly due to wide differences of growth rates in individual embryos. However, if we disregard the abnormally high titer at two days, the general trend of the growth curve becomes apparent. The initial amount of virus in the yolk sacs and the amount of virus during the lag period were lower than in the corresponding time intervals in living embryos and, the titer, though rising, remained considerably lower through the 5th day. At about the 6th day the titer was as high as the declining titer of the virus inoculated into living embryos and, by the 7th day, was considerably higher. The trend of the growth curve was probably determined by the low concentration of virus at the start. It resembles the type of curve one would expect to find when virus has been inoculated into embryos in small concentrations.

The growth curve of D18 started with a high concentration, reached a peak at 4 days, and declined slowly afterwards. It resembled that of the original strain in living embryos, except that a lower rate of growth and peak were reached.

At the peak of the infection each gram of yolk sac of either group of dead embryos contained approximately 300 times the total amount injected into each embryo. The magnitude of the differences in virus content among individual embryos in each series is shown by the wide difference in virus content immediately after inoculation of a same amount of virus. Therefore, only the over-all trend of the growth curves should be considered significant. The virus seemed to fare somewhat better in living embryos than in dead ones.

DISCUSSION

The foregoing results clearly indicate that the agent of feline pneumonitis can grow in the yolk sacs of dead chick embryos. An equilibrium was reached after the first few passages, and thereafter the virus seemed capable of indefinite passage. Eighteen passages in dead embryos did not result in any detectable changes in the properties of the virus with respect to morphology, virulence for the chick embryo or mouse, and antigenicity. These findings are in marked contrast to the changes in virulence for mice between the strain growing in mice and the same strain after repeated passages in chick embryos.

The difference in rates of growth in living and dead embryos appeared small. The advantage of living embryos over dead ones for virus growth may have been due only to mechanical causes. For example, the movements and circulation of the living embryos may have spread the inoculum more evenly over the yolk sacs and therefore may have greatly increased the surface of attack by the virus. Growth of the virus in dead embryos, on the other hand, depended upon the amount of virus initially deposited on a suitable site of development.

Differences between the growth curves of the original and D18 strains in dead embryos were not sufficiently great to indicate that an adaptation had occurred in D18.

The results show that metabolic rates, if they are different in the host

cells of dead embryos, do not appreciably affect the rate of viral growth. However, it would be unwarranted to assume further that the virus may not be affected by changes in host cell metabolism. The metabolism of the host cells of dead embryos has been compared to that of cells from living embryos by Moulder and Weiss (1950). In this study it was found that the rates of oxygen uptake and anaerobic glycolysis of yolk sacs from dead embryos were not greatly different from the rates in yolk sacs of living embryos during the period in which virus growth was demonstrated. Therefore, only the influence of the embryo as a whole was radically changed, and the yolk sacs became similar to tissue culture mediums.

SUMMARY

The agent of feline pneumonitis was cultivated for 20 passages in the yolk sacs of dead chick embryos. The strain obtained from the 17th passage appeared identical to the original strain with respect to morphology, virulence for chick embryos and mice, and antigenicity. The rates of growth in living and dead embryos were very similar, although a somewhat higher titer was reached in living embryos.

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STUDIES ON THE EFFECTS OF C_{18} UNSATURATED FATTY ACIDS ON GROWTH AND RESPIRATION OF *MICROCOCCUS PYOGENES* VAR. *AUREUS*

E. STATEN WYNNE AND JACKSON W. FOSTER

From the Department of Plant Sciences, University of Oklahoma, Norman, Oklahoma and the Department of Bacteriology, University of Texas, Austin, Texas

The inhibitory effects of C_{18} unsaturated acids toward various bacterial species have been subjects of numerous investigations (see Humfield[1] for summary of literature). The toxicity of linoleic acid against growth of *Lactobacillus helveticus* was readily reversed by lecithin, cholesterol, lumisterol, calciferol, and α-tocopherol in studies by Kodicek and Worden.[2] Stearic, malic, and oxalacetic acids were somewhat less effective, while ergosterol and sodium taurocholate were devoid of reversing action.

Appropriate concentrations of oleic, linoleic, and linolenic acids have been reported to depress growth of *Micrococcus* species by Frobisher,[3] Kodicek and Worden,[2] Dubos,[4] and Humfield.[1] The present investigation represents chiefly at attempt to extend the reversal studies of Kodicek and Worden[2] to *Micrococcus pyogenes* var. *aureus*, which limited investigations of the effect of linolenic acid on respiration.

EXPERIMENTAL

The Rosenbach strain of *M. pyogenes* var. *aureus* was used throughout this work. For the growth experiments a basal medium was employed with the following composition per liter: casamino acids, Difco vitamin-free, 2 g; glucose, 2 g; biotin, 5 μg; nicotinamide, 1 mg; thiamin, 200 μg; K_2HPO_4, 0.015 *M*; KH_2PO_4, 0.015 *M*; $CaCl_2$, 0.0001 *M*; $FeSO_4 \cdot 7H_2O$, 0.00005 *M*; $MnSO_4 \cdot 2H_2O$, 0.0001 *M*; $MgSO_4 \cdot 7H_2O$, 0.0002 *M*; $NaHCO_3$, 0.02 *M*; pH 7.2 to 7.4. The fatty acids used were added in the form of sodium salts, and the final volume was 10 ml per 25×150 mm tube.

For preparation of inoculums the organism was grown for at least two transfers in the basal medium. One drop of an 18- to 24-hour culture was then used per tube.

Incubations were at 37 C, and growth was estimated turbidimetrically by means of an "Aminco" photoelectric colorimeter at a wavelength of 530 mμ. Preliminary readings of varying dilutions of a full-grown culture showed that log L_0/L plotted against the concentration of bacteria yielded a straight line; i.e., the Bouger-Beer Law[5] was obeyed over the ranges employed. The higher log values indicate heavier growth. Zero time turbidities were generally determined by averaging four replicates, while readings of incubated tubes usually represented averages of duplicates.

For studies of oxygen uptake by use of the Warburg respirometer, cells grown 18 to 24 hours on nutrient agar with 0.2% added glucose were harvested in *M*/50 phosphate buffer at pH 7,

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washed once, and resuspended in the buffer in such concentration that a 1.10 dilution gave a reading of approximately 27 in the densitometer.

Sodium oleate was used in all growth experiments, though later sodium linolenate proved to be approximately three times as effective in inhibiting multiplication. Stock 1% aqueous solutions of the soap were found not to decrease significantly in strength after one month at 9 C. Typical effects of 30 μg/ml and 90 μg/ml of oleate are shown in figure 1. Considering the differences in experimental conditions, the degree of inhibition generally found with oleate was in rough agreement with the finding of Humfield[1] that 23 μg/ml gave 50% inhibition at 4 hours incubation.

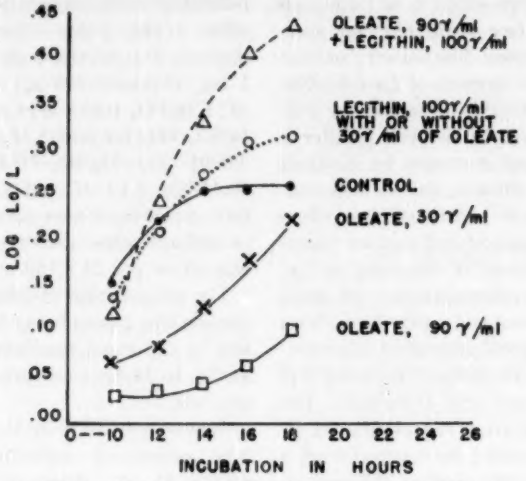

FIG. 1.—Toxicity of oleate and its reversal by lecithin.

The reversing effects of 100 μg/ml of lecithin on the toxicity of oleate are also shown in figure 1. Not only was the inhibition due to oleate completely overcome, but lecithin plus 90 μg/ml of oleate gave considerably greater stimulation than lecithin alone. The validity of these results was borne out by data (not shown) for 60 μg/ml of oleate, which alone gave a degree of inhibition intermediate between those obtained for 30 μg/ml and 90 μg/ml, and when combined with 100 μg/ml of lecithin gave a stimulation curve lying approximately half-way between those for the two concentrations shown in figure 1. Furthermore, 10 μg/ml of lecithin exerted definite, though partial, reversal of the inhibitory effects of 30, 60, and 90 μg/ml of oleate. One μg/ml of lecithin was without effect.

Cholesterol differed from lecithin in being quite toxic itself at a concentration of 100 μg/ml (fig. 2). A mutual detoxification appeared to occur between cholesterol and 90 μg/ml of oleate, since growth in the presence of both was considerably better than that with either alone (fig. 2). Similar results were obtained with 100 μg/ml of cholesterol and 30 μg/ml of oleate. With 30 μg/ml of cholesterol no apparent toxicity was observed, but this concentration completely reversed the inhibitory effect of 30 μg/ml of oleate and partially reversed that of 90 μg/ml. At 10 μg/ml, cholesterol had no reversing action.

An interesting effect consistently noted with cholesterol was a notable absence of additive turbidity. In fact, a simple solution of cholesterol plus oleate usually gave a greater light transmission than that of either alone. Thus in one experiment 100 μg/ml of cholesterol gave a reading of 43, 30 μg/ml of oleate a reading of 80, while both read 88. Such findings were interpreted as indicative of mutual solubilization of some sort.

Ergosterol, which is structurally quite similar to cholesterol except for the presence of two additional double bonds and an additional methyl group, was nevertheless much more toxic. Whereas cholesterol was less inhibitory than oleate, ergosterol was several times more toxic (table 1). Ergosterol in a final concentration of 100 μg/ml completely prevented growth even after an observed incubation period of 51 hours, in contrast to the report by Frobisher[3] that 1000 μg/ml of oleate gave only partial inhibition of 4 out of 5 strains of *Staphylococcus aureus* after 48 hours. Addition of sufficient concentrations of oleate diminished the toxicity of ergosterol, but resulting growth never exceeded that with oleate alone (table 1).

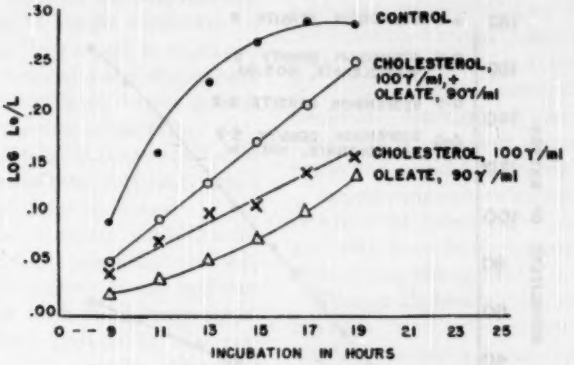

FIG. 2.—Mutual detoxification of oleate and cholesterol.

Cholic acid, which is structurally somewhat similar to the two sterols, was tested in concentrations of 10, 30, and 100 μg/ml, both alone and in the presence of oleate. It was entirely nontoxic, however, and also failed to exert any measurable reversing action toward oleate inhibition. Incidentally, cholic acid failed to affect the light transmission of the medium.

In studies with the Warburg respirometer, sodium linolenate was used throughout. A final concentration of 100

TABLE 1.—*Effect of oleate on ergosterol toxicity.*

Incubation (hours)	Oleate (μg/ml)	Ergosterol, μg/ml					
		0		30		100	
		Colorimeter reading	Log L_0/L	Colorimeter reading	Log L_0/L	Colorimeter reading	Log L_0/L
0	0	98		97		88	
	30	80		80		81	
	90	69		66		74	
17	0	50	0.29	80	0.08	88	0.00
	30	50	0.20	60	0.12	69	0.07
	90	55	0.10	53	0.10	63	0.07

μg/ml was observed to abolish effectively the low but appreciable endogenous oxygen uptake. In the presence of glucose the inhibitory effect of linolenate on oxygen consumption was repeatedly observed to be dependent on the concentration of the cell suspension used (fig. 3).

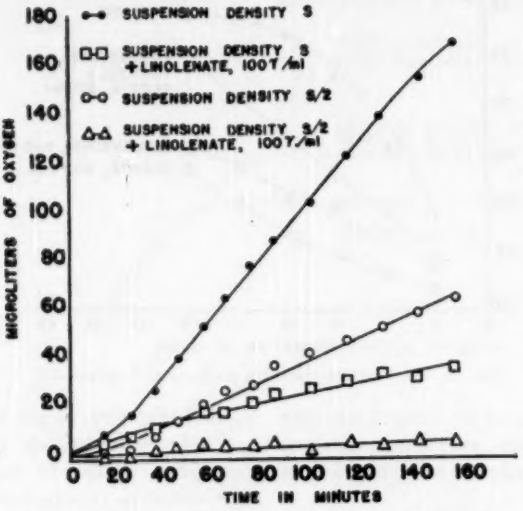

FIG. 3.—Effect of density of cell suspension on linolenate inhibition of oxygen uptake.

That the observed inhibition of respiration was due to a rapid bactericidal effect of linolenate is considered likely by the fact that plate counts from suspensions parallel to those used in the Warburg vessels showed that up to 90% of the cells were killed within 10 minutes.

DISCUSSION

The interrelationships of lipoidal materials noted here are by no means easily explained. We agree with Kodicek and Worden[5] that some sort of surface phenomena, perhaps affecting permeability, may be involved. Relevant in this connection is the view of Williams and Fieger[6] that various detergent materials, and perhaps even biotin, act by altering permeability. The marked effect of density of suspension on the depression of oxygen uptake noted with linolenate is compatible with a surface mode of action, since the amount of linolenate per cell would presumably be less in heavier suspensions. However, since all substances tested are surface active and yet differed in behavior, it may be surmised that surface action per se does not account for the results and that specific molecular structure is of importance.

The reversal of ergosterol toxicity by the less toxic sodium oleate may be visualized as a replacement of molecular or other units of the former by those of the latter at the cell membrane, which is generally supposed to be lipoprotein in nature. Recently the highly surface-active "Tween 80" has been reported by Eiseman[7] to decrease the toxicity of cer-

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tain surface-active drugs for *Mycobacterium tuberculosis* by at least 1000-fold, presumably by such a mechanism. A replacement of ergosterol by oleate would be favored by a stabilizing effect of oleate on colloidal ergosterol micellae, and the mutual solubility effect of the two compounds from densitometer data is compatible with such a stabilization.

The reversal of oleate inhibition by cholesterol may, in general, be explained similarly, but here mixtures of appropriate concentrations gave better growth than that occurring with either substance alone. These findings emphasize the importance of even relatively minor changes in molecular structure.

The reversal of oleate inhibition by nontoxic lecithin proceeded a step further in that growth in selected concentrations of both substances significantly exceeded growth in the controls. Lecithin is known to react with acids[8] and at autoclave temperature could conceivably form a complex with oleate that might be stimulatory. Complex formation has been invoked by Kodicek and Worden[2] to explain the reversing action of both lecithin and cholesterol on linoleate inhibition of *L. helveticus*. Analogous effects of serum albumin on oleate inhibition of *Myco. tuberculosis* have been reported by Dubos.[4]

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SUMMARY

Sodium salts of oleic and linolenic acids were quite inhibitory to growth of the Rosenbach strain of *Micrococcus pyogenes* var. *aureus* in a synthetic medium. Somewhat higher concentrations of linolenate markedly depressed oxygen uptake of suspensions, presumably because of a rapid bactericidal effect.

Appropriate concentrations of lecithin reversed the growth inhibition of oleate and even gave better growth than was obtained in the controls.

Though cholesterol was somewhat inhibitory itself, oleate and cholesterol displayed a mutual detoxifying action. Ergosterol was much more toxic, but oleate was capable of reducing the toxicity of ergosterol to that observed with oleate alone.

Cholic acid, though structurally similar to the sterols, was nontoxic and likewise failed to exert a reversing action on inhibition due to oleate.

The significance of these findings is discussed.

MUCOLYTIC ENZYME SYSTEMS. IX. NONSPECIFIC HYALURONIDASE INHIBITOR IN RHEUMATIC FEVER

ROBERT A. GOOD* AND DAVID GLICK

From the Departments of Pediatrics and of Physiological Chemistry, University of Minnesota Medical School, Minneapolis, Minnesota

While neither the specific etiology nor the pathogenic mechanism that results in acute rheumatic fever is known with certainty, extensive clinical, bacteriological, and immunological studies have implicated the β-hemolytic streptococcus as the trigger that sets into operation a chain of events which results in this well known but perplexing clinical syndrome.[1–6] Concepts of the pathogenic processes initiated by the streptococcus and resulting in rheumatic fever are notable for their variability. Theses implicating direct streptococcus invasion[7] and allergic[8,9] and autoallergic[10] functions have been proposed as possible solutions to this problem. Recently, speculation has led to the consideration of the pathological activity of substances produced or secreted by the streptococcus.[11] Meyer [12] has called attention to the intriguing possibility that hyaluronidase in or liberated by the invading streptococcus might be playing an important role in the pathogenesis of rheumatic fever by initiating injury of the hyaluronic acid rich synovial tissues and connective tissue in general.

Studies emphasizing the diffuse connective tissue disturbance in rheumatic fever[13–15] as well as evidence that group

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The assistance of Dr. M. J. Shapiro and Dr. Paul F. Dwan in acquisition of clinical material and selection of patients is gratefully acknowledged.

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A β-hemolytic streptococci can produce hyaluronidase[16] are in keeping with this hypothesis. Investigation of the specific antihyaluronidase titers in normal children and children suffering from acute streptococcus infection or rheumatic fever[17–19] would seem to indicate that hyaluronidase regularly gains entrance into the body in association with streptococcus infection, particularly preceding rheumatic sequellae. Hyaluronidase, consequently, could be playing some part in the pathogenesis of rheumatic fever.

In addition to and distinct from the specific antihyaluronidase antibody, there exists in the blood of normal and diseased individuals a nonspecific inhibitor of hyaluronidase. This hyaluronidase inhibitor differs from the antibody against hyaluronidase in that the former has a greater heat lability, lower electrophoretic charge density, nonspecific inhibiting effect on hyaluronidase from different sources, and an earlier increase of the blood level in disease processes. The occurrence of nonspecific hyaluronidase inhibitor in human serum under a variety of conditions has been studied by Dorfman and coworkers[20–22] and Glick and coworkers[23–28] and has been shown to increase in proportion to the severity of the involvement in generalized malignancy as well as in bacterial and virus infections.

Although Fulton et al[29] observed no difference in the degree of enzyme inhibition by the serums of normal individuals and patients with rheumatic

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arthritis, Grais and Glick[30] found marked elevation of this substance in their one case of active rheumatic fever. In the light of the possible role of hyaluronidase in the pathogenesis of rheumatic fever and the consequent significance of all the bodily defenses against hyaluronidase action, a study of the alterations in the nonspecific hyaluronidase inhibitor in rheumatic fever was undertaken. Serums from normal young adults, normal children from 1 to 15 years of age, and children with active and inactive rheumatic fever, Sydenham's chorea, and streptococcus pharyngitis were studied.

MATERIALS AND METHODS

The preparation of the hyaluronic acid substrate obtained from human umbilical cords and the bovine testicular hyaluronidase used, as well as the details of the viscosimetric method employed for quantitation of the hyaluronidase inhibitor activity, have been reported in previous publications of this series.[23,31] The degree of hyaluronidase inhibition is expressed as the percent inhibition. The percent inhibition was defined as $100(R-Ro)/R$, where Ro is the time required to reduce the viscosity of the reaction mixture to one-half its original value, and R is the time required to reduce the initial viscosity to one-half after incubation of 0.5 cc of enzyme solution with 0.02 cc of serum and 1.48 cc of water for 10 minutes at 37.5 C.

All specimens of blood were obtained by venipuncture, allowed to clot at 4 C for one hour, and centrifuged at 2500 to 3000 revolutions per minute for 15 to 20 minutes. The serum was then poured off and stored at −30 C until used. Specimens from normal and diseased patients were collected and studied simultaneously to obviate variabilities inherent in storage as well as enzyme and substrate fluctuations. Blood specimens from 25 healthy adults ranging in age from 18 to 48 were obtained from members of the medical and nursing staffs at the University Hospital. The fifty specimens from normal children were obtained from among patients ranging in age from 1 to 15 years presenting themselves to a large outpatient service for dental care, eye refraction, minor congenital anomalies, and psychogenic behavior disturbances. Careful histories as well as thorough physical and routine laboratory examinations revealed no evidence of recent or existing disease at the time the blood specimens were drawn from these patients.

For comparison with these healthy children and adults, blood from patients with acute, convalescent, and inactive rheumatic fever, uncomplicated Sydenham's chorea, and acute streptococcus pharyngitis was obtained. All patients with acute rheumatic fever studied were suffering with fever, polyarthritis, and carditis. In each case, marked elevation of the erythrocyte sedimentation rate was demonstrated. Additional rheumatic manifestations including subcutaneous nodules, skin rashes, and chorea were noted in some of the children. No cases in which the diagnosis of acute rheumatic fever was not reasonably certain were included. In a few cases only a single blood specimen was obtained, but in most cases serial samples of blood were studied throughout the rheumatic episode. Following the return of these same patients to rheumatic quiescence, a series of samplings was obtained for study of the hyaluronidase inhibitor content of the serum in convalescent rheumatic fever.

The patients with inactive rheumatic fever were those presenting a history of previous acute rheumatic fever and sustaining residual valvular cardiac damage as evidenced by characteristic heart murmurs. Interval medical histories, physical examinations, and laboratory studies revealed no other evidence of disease at the time this study was carried out. In addition, a group of 14 patients with severe Sydenham's chorea uncomplicated either by other manifestations of active rheumatic disease or intercurrent infection and a small group of patients with acute uncomplicated streptococcus pharyngitis were studied. Serums from several patients with inactive rheumatic fever complicated by nonstreptococcal upper respiratory infection were also studied.

No medications were given to any of the patients included in this study before the initial blood samples were drawn. The patients suffering with acute rheumatic fever were treated with moderate, symptom-relieving dosages of salicylates in the form of either acetyl salicylic acid

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TABLE 1.—*Hyaluronidase inhibitor in normal and rheumatic children.*

Group	Number of cases	Age	Mean percent inhibition	S.E. of mean
Normal adults	25	18 to 48	20.3	±1.48
Normal children	8	1 to 3	22.1	±1.53
	9	4 to 6	26.0	±1.98
	16	7 to 11	20.3	±1.09
	17	12 to 18	20.0	±1.34
	50	1 to 18	21.5	±0.714
Males	34	1 to 18	21.7	±1.06
Females	16	1 to 18	20.4	±1.08
Acute rheumatic fever	20	4 to 22	41.8	±2.39
Convalescent rheumatic fever	10	4 to 15	12.9	±1.13
Inactive rheumatic fever	50	5 to 20	15.7	±0.760
Sydenham's chorea	14	7 to 14	16.5	±1.80
Streptococcus pharyngitis	10	3 to 15	31.2	±4.6
Inactive rheumatic fever complicated by U.R.I.	11	7 to 16	25.3	±1.3

or sodium salicylate during the period of clinical activity of the rheumatic episode. The patients who had acute chorea uncomplicated by other rheumatic symptomatology were treated with bed rest and occasional moderate dosages of barbiturates. Neither the children in the "convalescent" rheumatic fever or "inactive" rheumatic fever groups were being given any medication at the time of this study. Following the initial bleeding, the patients with streptococcal pharyngitis were treated either with intramuscular injections of aqueous penicillin or orally administered sulfadiazine in therapeutic dosages until the symptoms of infection had abated. Grais and Glick[31] have shown that neither the sulfa drugs nor penicillin in therapeutic dosages influence the hyaluronidase-inhibitor value as measured in this study.

RESULTS

The results of these studies are summarized in table 1. It is apparent from the data presented that the nonspecific hyaluronidase inhibitor in the serums of normal children does not differ significantly from that of healthy young adults. The data broken down with respect to age groups of children do not reveal noticeable variation. Marked increase of serum hyaluronidase inhibition was apparent in the acute exudative phase or rheumatic fever. Approximately 100% increase of inhibitor activity was noted in patients with active rheumatic fever when compared to the levels obtained for normal children. Comparison of the active rheumatic group with the patients having inactive rheumatic fever reveals this difference to be in the neighborhood of 200%. In no case of active rheumatic fever was the hyaluronidase inhibitor level as low as the mean for the normal children. The general impression was gained that the elevation of hyaluronidase inhibitor was roughly proportional to the clinical severity of the exudative process of rheumatic fever as indicated by the joint involvement, fever, sedimentation rate, and carditis. In figures 1, 2, and 3, representative curves of the hyaluronidase inhibitor level plotted against time during an episode of rheumatic fever are compared with similar recordings of temperature and sedimentation rate. These data taken throughout the rheumatic episodes would tend to confirm the impression gained from the study of the group of patients with active rheumatic fever that the degree of exudative rheumatic activity was correlated with the degree of elevation of the hyaluronidase inhibitor.

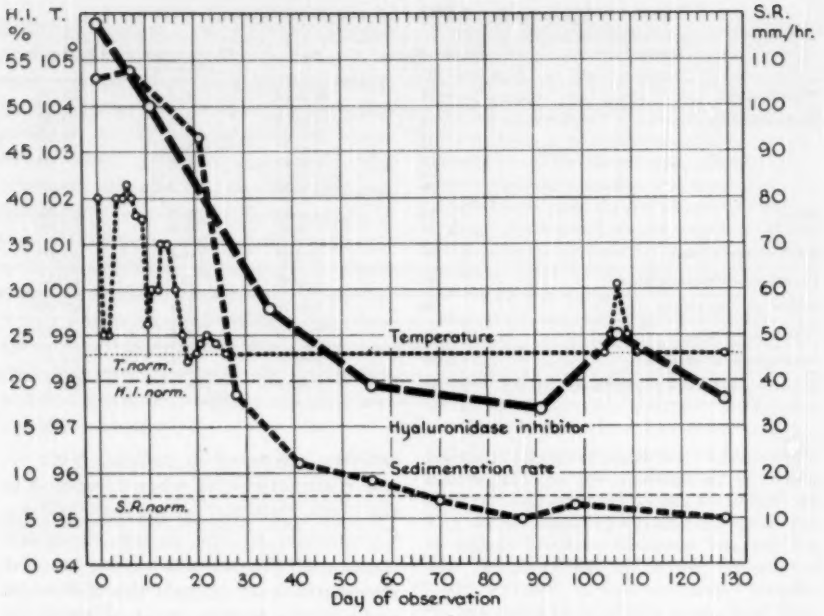

FIG. 1.—B.K. Hyaluronidase inhibitor levels determined serially during an episode of acute rheumatic fever. Parallel fall in erythrocyte sedimentation rate, fever, and hyaluronidase-inhibitor value accompanying clinical recovery are shown.

From table 1 it would further seem that patients convalescent from rheumatic fever have lower concentration of serum hyaluronidase inhibitor than do the normal healthy children. This same deficiency perhaps to a lesser degree is apparent in the case of the children with inactive rheumatic fever. The patients with a actue severe Sydenham's chorea not only show no consistent elevation of serum hyaluronidase inhibitor as measured in this way, but the mean value obtained for this group is comparable to that obtained for the convalescent and inactive rheumatic fever patients and somewhat lower than the normal values.

Insufficient data are available to permit final conclusions with regard to the hyaluronidase inhibitor values in streptococcal infection. In the small series of cases of acute streptococcal pharyngitis studied, however, wide variation of inhibitor levels was noted.

As may be seen in table 1, the small group of patients suffering from acute β-hemolytic streptococcus pharyngitis showed a significant elevation of the mean level of serum hyaluronidase inhibitor. This finding is in keeping with previous reports from this laboratory of elevations of hyaluronidase inhibitor during other bacterial or virus infections. Of particular interest with regard to the finding reported here, that marked elevation of hyaluronidase inhibitor occurs in the serum of patients during episodes of acute rheumatic fever, was the additional finding that normal or near-normal levels of hyaluronidase inhibitor occurred in the serums of five patients studied two or three weeks after recovery from a pharyngeal

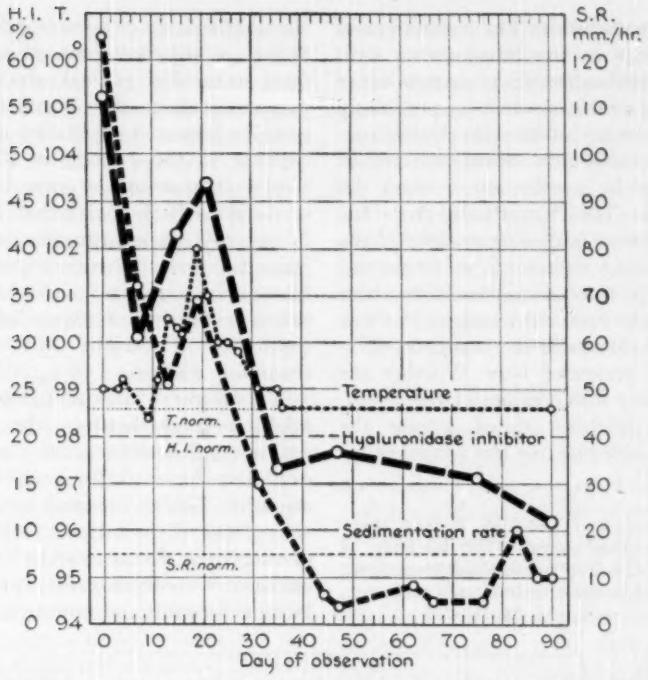

FIG. 2.—S.G. Hyaluronidase inhibitor levels in an episode of acute rheumatic fever. Here a clinical exacerbation is associated with coincident elevations of fever, sedimentation rate, and hyaluronidase inhibitor. Return of hyaluronidase inhibitor to subnormal level in convalescence is shown.

infection with β-hemolytic streptococcus. Two additional patients, whose serums were collected both during an acute scarlet fever infection and two to three weeks following recovery, showed elevations of hyaluronidase inhibitor while the patients were acutely ill with a return toward normal levels in the convalescent period. Although this case material is obviously scanty, the findings are in keeping with the earlier reports in this series that serum hyaluronidase inhibitor is elevated during the acute phase of bacterial or virus infections and returns promptly to normal levels as the infection subsides.

DISCUSSION

Previous studies have shown that the nonspecific hyaluronidase inhibitor[22–25,30] does not vary with the age of the patient from 16 to 80, and our present studies indicate, in addition, that in childhood no significant variation with age occurs. Dorfman[21] reported that males of reproductive age have significantly lower inhibitor levels than any other group studied. This is in contrast to the data presented in the present paper as well as the previous publications in this series which showed no significant variation with age or sex. The marked elevation of hyaluronidase inhibitor in the exudative phase of rheumatic fever as well as in acute bacterial and virus infections and disseminated malignancy indicates that this biological response

is truly nonspecific. The correlation of this change in the blood serum with severity of the destructive process under these circumstances and, as previously noted,[32] during uterine involution suggests that inhibitor elevations may in some way be a reflection of tissue destruction. The hypothesis that the hyaluronidase-inhibiting activity of the serum might represent an important nonspecific defense reaction of the host has already been discussed in previous papers of this series and is supported by the data presented here. Whether the consistently lower values of the hyaluronidase inhibitor noted among the patients with inactive and convalescent rheumatic fever have a special significance is still unknown. It is certain from extensive clinical studies that patients such as those studied in this group represent a population more susceptible to the ravages of rheumatic disease than groups of normal children or adults. If hyaluronidase activity plays a role in the pathogenesis of rheumatic fever, the differences in serum inhibitor concentration might have considerable bearing on the relative susceptibility of various individuals to rheumatic disease.

The difference in serum hyaluronidase inhibitor levels between the patients with acute Sydenham's chorea and acute exudative rheumatic fever requires some attention. Chorea, although usually considered one of the major manifestations of rheumatic disease, rarely shows the changes in sedimentation rate, white blood cell count, and temperature asso-

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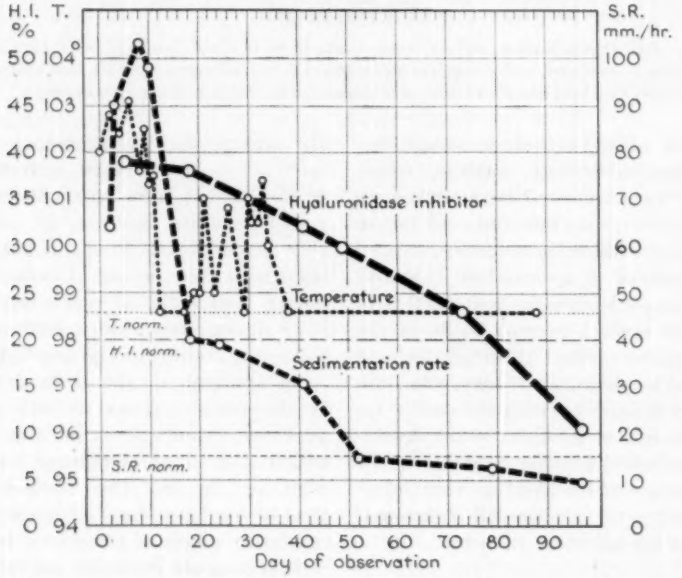

FIG. 3—G.A. Hyaluronidase inhibitor in acute rheumatic fever. The persistent elevation of hyaluronidase inhibitor throughout clinical activity of the rheumatic process and the return to subnormal levels in convalescent period are illustrated.

ciated with carditis, polyarthritis, and subcutaneous nodule formation. The clinical difference between these components of this protean disease is further emphasized by the lack of alteration in the serum levels of hyaluronidase inhibitor in acute chorea uncomplicated by the other manifestations of rheumatic fever. A similar relationship of this manifestation of the rheumatic syndrome to biochemical changes was noted with respect to the serum mucoproteins.[33]

The findings with respect to the alteration of hyaluronidase inhibitor during acute exudative rheumatic fever might be clinically useful. The parallelism between sedimentation rate, temperature, and hyaluronidase inhibitor, changes which have been found to hold in the cases studied, is in accord with the correlation previously reported[30] for these three measurements. While its lack of specificity limits the effectiveness of this test as a diagnostic procedure, the data presented would suggest that the serum concentration of hyaluronidase inhibitor is a good index of the degree and duration of clinical rheumatic activity in many patients. The relationship of the alterations of this serum component to other mechanisms of adaptation to disease and the defense capacities of the host needs further investigation. A linkage of nonspecific hyaluronidase inhibitor with certain fractions of complement has already been demonstrated[34,35] and its relationship to the mucoprotein changes of the serum has been previously discussed.[36] That certain complement fractions enter into the inhibitor action is possible, but the marked elevation of inhibitor in acute rheumatic fever in contradistinction to the depressed or normal complement levels reported for the same disease would indicate that the two substances are not identical. Further research on this problem must be done before final conclusions as to the actual relationship of these substances can be drawn.

SUMMARY

1. The nonspecific hyaluronidase inhibitor of the serum of normal adults and children was compared to that of children with active, convalescent, and inactive rheumatic fever, Sydenham's chorea, and streptococcus pharyngitis.

2. Significant elevation of hyaluronidase inhibitor was found in acute exudative rheumatic fever and in acute streptococcus pharyngitis, while low levels, even with respect to normals, were characteristic of the serum of the patients with convalescent and inactive rheumatic fever.

3. Patients with Sydenham's chorea uncomplicated by other rheumatic manifestations showed no elevation of hyaluronidase inhibitor in their serums.

4. The relationship of the hyaluronidase inhibitor level to the defense reactions of the host was discussed.

Since this manuscript was prepared for publication Dorfman has presented a paper at the Hyaluronidase Symposium in New York in which he described an elevation on nonspecific hyaluronidase inhibitor, as measured by the turbidimetric technique, in the early phases of rheumatic fever with subnormal inhibitor levels, occurring in the period of convalescence from this disease.

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IMMUNOLOGIC STUDIES WITH *MALLEOMYCES MALLEI* AND *MALLEOMYCES PSEUDOMALLEI*. I. SEROLOGICAL RELATIONSHIPS BETWEEN *M. MALLEI* AND *M. PSEUDOMALLEI*

LEO CRAVITZ* AND WINSTON R. MILLER†
From Camp Detrick, Frederick, Maryland

The development of serological techniques with *Malleomyces mallei* date back to 1896 when M'Fadyean applied the agglutination test for the diagnosis of glanders in horses. In 1909, Schutz and Shubert[1] published their results on the application of the *M. mallei* complement fixation test for the diagnosis of glanders in horses. The precipitin test was used in diagnosis of glanders by Pfeiler[2] in Germany, Konen[3] in Russia, and Boyd[4] in the United States, but it has not been applied extensively in practice due to the frequency of false positive reactions. In 1930, Sakamoto[5] extracted a soluble polysaccharide from *M. mallei* which was stated to give a specific precipitation with serum from infected animals.

The agglutination reaction has been used by Stanton and Fletcher[6,7] and Sherwood and Lan[8] in studying the antigenic relationships between *M. mallei* and *Malleomyces pseudomallei*. Review of their work indicated (1) that *M. mallei* strains were serologically heterogeneous, (2) that *M. pseudomallei* (*B. whitmori*) strains were serologically homogeneous, (3) that strains of *M. mallei* were sharply divided into two subgroups by their serological reactions, and (4) that one subgroup of *M. mallei* was very closely related to *M. pseudomallei*, whereas the other subgroup was only distantly related to *M. pseudomallei*.

MATERIALS

Strains of organisms.—Eight strains of *M. mallei* and 2 strains of *M. pseudomallei* were used in the present studies. Three strains of *M. mallei* were obtained in this country (2MP, 2023, and 2024), and 5 strains were obtained from China (C3, C4, C5, C6, and C7). The 2 strains of *M. pseudomallei* were obtained from India. Details of the source, cultural characteristics, and pathogenicity of these organisms are reported elsewhere.[9,10]

Preparation of antiserums.—Ten immune serums were prepared by inoculating a series of rabbits with each of the 8 strains of *M. mallei* and 2 strains of *M. pseudomallei*. The vaccines (agglutinating antigen) used contained about one billion organisms per ml, and each rabbit received a total of 11 ml by the subcutaneous, intraperitoneal, and intravenous routes over a period of 4 weeks. The rabbits were bled 2 weeks after the

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* Rochester General Hospital, Rochester, New York.

† Henry Ford Hospital, Detroit, Michigan.

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10. Miller, W. R., Pannell, L., Cravitz, L., Tanner, W. A. and Rosebury, T. 1948, J. Bact. **55**: 127–135.

final injection, and the serums from animals inoculated with the same strain were pooled.

Preparation of agglutinating antigen from M. mallei.—Heavily seeded beef extract glycerol agar plates were incubated at 37 C for 24 hours. The growth was harvested in 0.5% phenolized normal saline and introduced into flasks containing glass beads. The suspension was shaken gently until essentially homogeneous and then placed in the 37 C incubator for 2 to 4 days. The suspension was tested daily for viability and when sterile was filtered through coarse filter paper and bottled. For use in the test, the antigen was diluted to equal the turbidity of a 33 McFarland Nephelometer tube (approximately one billion organisms per ml).

Preparation of agglutinating antigen from M. pseudomallei.—This antigen was prepared in the same manner as the *M. mallei* antigen except that the organisms were suspended in formalinized (0.1%) saline instead of phenolized saline. This served to preserve the flagellar components of the antigen.

Preparation of complement-fixing antigen.—The 24-hour growth of heavily seeded plates was suspended in formalinized (0.5%) saline and allowed to stand at 37 C until sterile (about 4 days). The suspensions were centrifuged at high speed and the clear supernatant fluid was removed. The supernatant fluid was preserved with merthiolate in final concentration of 1:5000 and used as the complement-fixation antigen. The antigenic titers of the antigens prepared from the various strains varied from 1:40 to 1:1280. The anticomplementary titers were never greater than 1:10 and were usually less than 1:5. The dilution of antigen used in the complement fixation tests was twice the anticomplementary titer. It therefore contained several antigenic units.

METHODS

The antigenic relationships of the strains of *M. mallei* and *M. pseudomallei* were studied by cross agglutination, cross complement fixation, and agglutinin absorption tests.

Agglutination technique.—Serial twofold dilutions in saline, beginning with 1:5 and ending with 1:1280, were made of each serum to be tested, and 0.5 ml amounts were distributed into Kahn tubes. Antigen was added in 0.5 ml quantities, giving final serum dilutions of 1:10 to 1:2560. The tubes were shaken and incubated at 37 C for 24 hours.

The extent of agglutination was ascertained by means of reflected light, using a microscope lamp and mirror. Due to the fineness of clumping, a correct reading could not be made without a source of light rays which could be reflected from the particles and magnified in a concave mirror. Excessive agitation was avoided since the clumps were easily disrupted. The readings were recorded on the following bases:

4 plus—complete, large clumps, supernatant clear.
3 plus—definite clumps, supernatant slightly cloudy.
2 plus—definite clumps, supernatant cloudy.
1 plus—very small clumps visible, supernatant cloudy.
± —questionable clumping.
Negative—uniformly turbid, no clumping.

Agglutination tests were performed with each of the 10 immune rabbit serums against suspensions of the 8 strains of *M. mallei* and the 2 strains of *M. pseudomallei*.

Complement fixation technique.—The methods of preparing and titrating the sheep red blood cells, antisheep aboceptor, and guinea pig complement, and for titrating the antigen for antigenic and anticomplementary properties were the standard techniques used in serological laboratories. The complement fixation titration was performed as follows: Fifteen tubes were arranged for each serum sample tested. The quantitative serum dilutions were set up in the first 9 tubes, the 10th and 11th tubes were utilized for the anticomplementary controls of the serum and antigen, and the remaining 4 tubes were used as controls for the hemolytic system.

One set of the hemolytic system and antigen controls was sufficient for any number of tests undertaken at one time when the same materials were used. Known positive and negative serum controls were always included to further control the results of the test. All serums were inactivated in a water bath at 56 C for 15 minutes prior to the titrations.

After the final incubation, the racks were placed in the refrigerator overnight and the results of the titrations were read in the morning. In the tubes with positive "fixation" as well as in the complement, amboceptor, and saline controls, the blood cells settled to the bottom of the tube and the supernatant was water clear. The degree of fixation was recorded on a negative to 4 plus basis in the usual manner.

Complement fixation tests were performed with each of the 10 immune rabbit serums set up against the soluble antigens of the 8 strains of *M. mallei* and 2 strains of *M. pseudomallei*.

Agglutinin absorption technique.—Aliquot portions of immune rabbit serum prepared against *M. mallei*, strains C3, C4, C5, and C6, and *M. pseudomallei*, strains W294 and W295, were ab-

TABLE 1.—*Cross agglutination titers between several strains of* M. mallei *and* M. pseudomallei.

Antigen†	Rabbit antiserums									
	C3	C4	C5	C6	C7	2MP	2023	2024	W-294	W-295
C3	1:5120*	1:5120	1:640	1:1280	1:2560	1:320	1:160	1:640	1:1280	1:640
C4	1:5120	1:5120*	1:1280	1:320	1:2560	1:160	1:160	1:320	1:2560	1:1280
C5	1:1280	1:1280	1:5120*	1:2560	1:640	1:1280	1:2560	1:2560	1:2180	1:640
C6	1:640	1:320	1:2560	1:2560*	1:320	1:1280	1:1280	1:1280	1:320	1:640
C7	1:5120	1:5120	1:640	1:640	1:2560*	1:320	1:320	1:320	1:640	1:1280
2MP	1:1280	1:640	1:1280	1:2560	1:1280	1:1280*	1:640	1:1280	1:1280	1:2560
2023	1:640	1:640	1:2560	1:2560	1:640	1:1280	1:2560*	1:2560	1:1280	1:640
2024	1:640	1:640	1:1280	1:640	1:640	1:2560	1:2560	1:2560*	1:640	1:1280
W-294	1:1280	1:1280	1:640	1:1280	1:640	1:320	1:160	1:160	1:5120*	1:5120
W-295	1:1280	1:1280	1:640	1:320	1:640	1:160	1:160	1:160	1:5120	1:5120*

* Titer of antiserum against the homologous antigen.
† W-294 and W-295 are *M. pseudomallei;* others are *M. mallei.*

sorbed with each of the 6 antigens as follows: Heavy saline suspensions of killed organisms were centrifuged at high speed until they were solidly packed. One-half of 1 ml of the packed organisms was added to 2 ml of a 1:10 dilution of the antiserum. The tubes were thoroughly shaken and incubated for three hours at 37 C. The serum was then separated by centrifugation. The absorbed serums were then tested for agglutinins against their homologous antigens. Nonabsorbed homologous immune serum was used as a control in each series of tests.

RESULTS

Cross agglutination.—The results of the cross agglutination tests are summarized in table 1. The inherent limitations of the agglutination test do not permit a sharp differentiation of the strains based on cross agglutination data. However, it may be noted that the two *M. pseudomallei* strains are practically identical in their agglutination reactions, but that the *M. mallei* strains are dissimilar. It appears that strains C3, C4, and C7 are closely related and make up one group. Strains C5, C6, 2MP, 2023, and 2024 show some similarities but appear to form a heterogeneous group. Strains C3, C4, and C7 are more closely related antigenically to the *M. pseudomallei* strains than they are to the other *M. mallei* strains.

Cross complement fixation.—The re-

TABLE 2.—*Cross complement fixation titers between several strains of* M. mallei *and* M. pseudomallei.

Antigenic bacterial washings†	Rabbit antiserums									
	C3	C4	C5	C6	C7	2MP	2023	2024	W-294	W-295
C3	1:640*	1:640	1:80	1:40	1:640	1:80	1:40	1:80	1:160	1:160
C4	1:640	1:640*	1:80	1:80	1:1280	1:80	1:80	1:160	1:160	1:160
C5	1:160	1:160	1:2560*	1:2560	1:160	1:160	1:160	1:640	1:160	1:160
C6	1:80	1:80	1:2560	1:2560*	1:80	1:80	1:160	1:320	1:160	1:160
C7	1:640	1:640	1:80	1:40	1:1280*	1:40	1:40	1:80	1:640	1:320
2MP	1:80	1:80	1:320	1:320	1:80	1:320*	1:320	1:640	1:80	1:160
2023	1:80	1:40	1:320	1:640	1:80	1:160	1:320*	1:1280	1:80	1:80
2024	1:40	1:80	1:640	1:320	1:80	1:80	1:640	1:1280*	1:80	1:80
W-294	1:640	1:320	1:80	1:80	1:320	1:80	1:80	1:80	1:640*	1:320
W-295	1:640	1:320	1:80	1:80	1:640	1:80	1:160	1:160	1:640	1:320*

* Titer of antiserum against the homologous soluble antigen.
† W-294 and W-295 are *M. pseudomallei;* all others are *M. mallei.*

sults of the cross complement fixation tests, summarized in table 2, are comparable to those obtained with cross agglutination. However, a more detailed grouping is indicated. Strains C3, C4, and C7 fall into one group which is more closely related to the group of *M. pseudomallei* strains than to the other *M. mallei* strains. On the basis of similar reactions, strains 2MP, 2023, and 2024 comprise a second *M. mallei* group, and C5 and C6 comprise a third group. The close interrelationship of individual strains in the first group of *M. mallei* and strains of *M. pseudomallei* shows a complete lack of species specificity.

Agglutinin absorption.—The summarized results of agglutinin absorption tests given in table 3 serve to substantiate the grouping and interrelationships of strains observed in cross agglutination and cross complement fixation studies. The similarity between C3 and C4, between C5 and C6, and between W294 and W295 is clearly demonstrated. C3 and C4 are again shown to be more closely related antigenically to W294 and W295 than to the other *M. mallei* strains C5 and C6.

Agglutination with O and OH antigens.—Cross agglutination titrations performed with whole (OH) and somatic (O) antigens of *M. pseudomallei* (W294) and antigens of *M. mallei* (C3), using immune rabbit serums prepared against the whole antigens. O antigens of *M. pseudomallei* were prepared by extraction of bacterial suspensions with 95% ethyl alcohol for 24 hours at 37 C and resuspending the centrifuged organisms in saline. Although *M. mallei* is not flagellated, alcohol-treated antigens were prepared as controls.

The results are given in table 4. The alcohol-treated antigen of *M. mallei* gave the same titers as the untreated antigen, and therefore only the former is presented. *M. pseudomallei* antiserum

TABLE 3.—*Agglutination titers with absorbed serums.*

C3 serum absorbed with	Titrated against C3 antigen	C4 serum absorbed with	Titrated against C4 antigen	C5 serum absorbed with	Titrated against C5 antigen	C6 serum absorbed with	Titrated against C6 antigen	W-294 serum absorbed with	Titrated against W-294 antigen	W-295 serum absorbed with	Titrated against W-295 antigen
C3	1:40*	C3	1:40	C3	1:2560	C3	1:640	C3	1:160	C3	1:320
C4	1:40	C4	1:20*	C4	1:1280	C4	1:1280	C4	1:80	C4	1:320
C5	1:2560	C5	1:1280	C5	1:40*	C5	1:20	C5	1:640	C5	1:640
C6	1:2560	C6	1:2560	C6	1:160	C6	Negative	C6	1:640	C6	1:1280
W-294	1:1280	W-294	1:1280	W-294	1:1280	W-294	1:640	W-294	1:20*	W-294	1:40
W-295	1:640	W-295	1:1280	W-295	1:1280	W-295	1:1280	W-295	1:40	W-295	1:40*
C3 serum (nonabsorbed)	1:5120	C4 serum (nonabsorbed)	1:5120	C5 serum (nonabsorbed)	1:5120	C6 serum (nonabsorbed)	1:5120	W-294 serum (nonabsorbed)	1:1280	W-295 serum (nonabsorbed)	1:2560

W-294 and W-295 are *M. pseudomallei*; all others are *M. mallei*.
* Absorption was not quite complete.

TABLE 4.—*Somatic and flagellar agglutination.*

Serum	Antigen	Titer
Anti-pseudomallei	Pseudomallei OH	1:1280
Anti-pseudomallei	Pseudomallei O	1:320
Anti-pseudomallei	Mallei O	1:320
Anti-mallei	Mallei O	1:1280
Anti-mallei	Pseudomallei O	1:160
Anti-mallei	Pseudomallei O	1:80

agglutinated the somatic *M. pseudomallei* antigen to only 25% of its titer and cross agglutinated the *M. mallei* antigen to the same degree. *M. mallei* antiserum cross agglutinated *M. pseudomallei* antigens in only partial degree. It would appear, therefore, that cross reaction is largely a function of the somatic antigenic components.

Species specificity in the complement fixation test.—Soluble antigens were prepared in several ways in an attempt to develop a complement fixation antigen which showed some degree of species specificity. In addition to the soluble antigen previously described, commercial mallein, filtrates of heavy suspensions disrupted by agitation with glass beads for 48 to 72 hours, filtrates of heavy suspensions after sonic vibration treatment, and urea extracts ("farase")[11] were tested. All of these antigens gave strong complement fixation reactions with both homologous and heterologous antiserums. The cross reaction between *M. mallei* strains C3, C4, and C7 and *M. pseudomallei* strains W294 and W295 were complete with all antigens.

Boiling for one hour did not destroy the antigenicity of any of the above soluble antigens. It seemed apparent, therefore, that the heat stable active principle was of a nonprotein nature. Accordingly, a soluble, nonprotein fraction was extracted and chemically purified. The method of preparation, chemical characteristics and reactions in the complement fixation test are reported elsewhere.[12] This fraction was strongly antigenic and gave the same complete cross reaction observed with the other antigens. It is regarded as the principle antigenic factor in the complement fixation reactions of the genus.

The precipitin reaction.—All of the soluble antigens used in the complement fixation tests gave strong precipitin reactions. Cross reactions occurred to the same degree as in the complement fixation reaction. Occasional false positive reactions with normal serum were observed, and the precipitin test was therefore felt to be of less value than the other serological tests.

DISCUSSION

The three groups of *M. mallei* strains and one group of *M. pseudomallei* strains, with the interrelationships observed, indicate the presence of a number of separate antigenic components. However, no species specificity of reaction was found since one group of *M. mallei* strains was more closely related antigenically to *M. pseudomallei* strains than to other strains of its homologous species. The nonprotein chemical fractions obtained from each species were strongly antigenic but not species-specific. The study of somatic and whole antigen reactions suggested the possibility of a specific *M. pseudomallei* flagellar antigen. Attempts to produce a flagellar antigen were unsuccessful. In reviewing the results of the studies, it would seem that a species-specific *M. pseudomallei* antibody might be obtained by absorption of immune serum with a somatic antigen of *M. pseudomallei*. Further study along this line is indicated.

The 8 *M. mallei* strains and 2 *M. pseudomallei* strains were the only strains of this genus that could be procured at the time the studies were con-

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ducted. Whether the general groupings and interrelationships of strains observed would hold true after study of a large number of strains is a matter of conjecture. The findings correlate well with those reported by Stanton and Fletcher working with entirely different strains. This correlation adds emphasis to the results and suggests a constancy of the antigenic patterns of these organisms.

The agglutination and complement fixation techniques described were widely used in the course of the studies on glanders and melioidosis and provided the only certain means of diagnosis of 6 cases of human glanders that occurred among the laboratory workers.[13]

SUMMARY

The techniques of the agglutination and complement fixation tests with *Malleomyces mallei* and *Malleomyces pseudomallei* have been described in detail.

The antigenic relationships of 8 strains of *M. mallei* and 2 strains of *M. pseudomallei* were investigated by means of cross agglutination, cross complement fixation, and agglutinin absorption studies. The following relationships were observed: (1) The *M. mallei* strains fell into three groups: (a) C3, C4, C7; (b) C5, C6; (c) 2023, 2024, 2MP. (2) The two *M. pseudomallei* strains were almost identical serologically. (3) The *M. mallei* strains in group (a) were more closely related to the *M. pseudomallei* strains than to *M. mallei* groups (b) and (c).

The agglutination of *M. pseudomallei* was largely a function of its flagellar antigen. The cross agglutination with *M. mallei* was a function of common somatic antigenic factors.

Efforts to prepare a complement fixation antigen which would differentiate between *M. mallei* and *M. pseudomallei* were unsuccessful. A chemically purified nonprotein fraction which was antigenically active in the complement fixation reaction appeared to be common to the two species.

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IMMUNOLOGIC STUDIES WITH *MALLEOMYCES MALLEI* AND *MALLEOMYCES PSEUDOMALLEI*. II. AGGLUTINATION AND COMPLEMENT FIXATION TESTS IN MAN AND LABORATORY ANIMALS

LEO CRAVITZ* AND WINSTON R. MILLER†
From Camp Detrick, Frederick, Maryland

The agglutination and complement fixation tests for glanders were originally developed as an aid in the detection of chronically infected horses.[1] It has been reported that serum of infected horses gave positive agglutination tests[2] in 84% and positive complement fixation tests[3,4] in 97% of the samples. Because of the scarcity of reports on human glanders in the past 30 years, there is very little serological data on human cases in the literature. The agglutination with serums of normal individuals has been reported to be about 1:100 and in exceptional instances as high as 1:400.[5] In human glanders only a few cases had agglutination titers under 1:80.[6] Usually titers were significantly elevated above the "normal" and some reached 1:2000.[7–9] The complement fixation reaction used by Watson[10] and Sabolotny[6] consistently gave reliable diagnostic results.

In a monograph on melioidosis, Stanton and Fletcher[11] reported that the serum from a chronic human case agglutinated its autogenous strain to a titer of 1:4000. In two other chronic cases studied by them, the serum of one gave a titer of 1:1280 while the serum from the other would not agglutinate its autogenous strain in dilutions higher than 1:80. "Normal" agglutinins were reported to be a titer of 1:80 in healthy persons. Harris et al[12] have recently reported two cases of chronic melioidosis in which the peak agglutination titers were 1:640 to 1:1280, and one case in which the titer never rose above 1:80. These workers also report control tests on other serums: syphilis, 40; infective hepatitis, 4; pulmonary tuberculosis, 2; and other fevers, 10. In no instance did controls give a higher titer than 1:20. They were of the opinion that a titer of 1:80 is significant and a titer of 1:160 almost certainly diagnostic.

The present communication reports the application of agglutination and

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* Rochester General Hospital, Rochester, New York.

† Henry Ford Hospital, Detroit, Michigan.

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complement fixation tests for glanders and melioidosis in man and animals and attempts to evaluate them as practical diagnostic aids. The methods for conducting the tests have been described in detail in the preceding paper.

Tests with normal human serums

Agglutination and complement fixation tests for glanders and melioidosis were performed with serums from approximately 400 apparently normal individuals chosen at random from among the military personnel at Camp Detrick. The results are given in table 1.

TABLE 1.—*Summary of agglutination and complement fixation titrations with normal serums.*

Test	Total Number of serums	Percentage of samples giving the following titers						
		Negative*	1:10	1:20	1:40	1:80	1:160	1:320
Agglutination, *M. mallei*	403	1.7	7.2	17.3	23.8	31.5	15.4	3.0
Agglutination, *M. pseudomallei*	419	2.9	7.6	16.9	24.3	29.4	16.2	2.6
Complement fixation	400	100						

* Negative at 1:10.

It is apparent that approximately 97 to 98% of normal individuals have agglutinins for both *Malleomyces mallei* and *Malleomyces pseudomallei*. The highest titer observed in this group was 1:320. Approximately 95% of the serums tested showed positive titers varying from 1:10 to 1:160. Only 3% gave a titer of 1:320 while 2 to 3% were negative (less than 1:10). The distribution of the titers for *M. mallei* paralleled those for *M. pseudomallei*.

The complement fixation tests with 400 samples of normal serums were all negative at a dilution of 1:10. Obviously, normal serums do not give positive complement fixation even in low dilution with the technique used.

Tests with serums from human cases of glanders

In a period of less than one year, 5 cases of human glanders developed as a result of infection during laboratory research. In 5 cases there was clinical and/or roentgenological evidence of patchy pneumonic consolidation resembling atypical pneumonia. The 6th case showed clinical evidence of splenomegaly and perisplenitis. All cases were diagnosed by the serological tests herein described. Sulfadiazine therapy resulted in marked clinical improvement and all cases eventually recovered completely.[13] The value of sulfadiazine therapy was first demonstrated in experimental glanders and melioidosis in animals by Miller et al.[14]

The serological findings in these cases together with pertinent clinical data are presented in figures 1 through 6. The diagnosis in all cases was based largely on a significant rise in serum agglutinins for *M. mallei*. The complement fixation test became positive in all of the cases except #6. It is possible that in this case the infection was arrested by prompt sulfadiazine therapy, which may account for a lack of a significant increase in circulating antibodies. Patient #3 showed a moderately high cross agglutination with *M. pseudomallei*, but the consistently higher titer against *M. mallei* indicated that the infection was more probably due to this organism.

In cases 4 and 5 a perceptible rise in the agglutination titer occurred in 2 to 3 weeks subsequent to infecti , followed by a progressive rise to 1:1280

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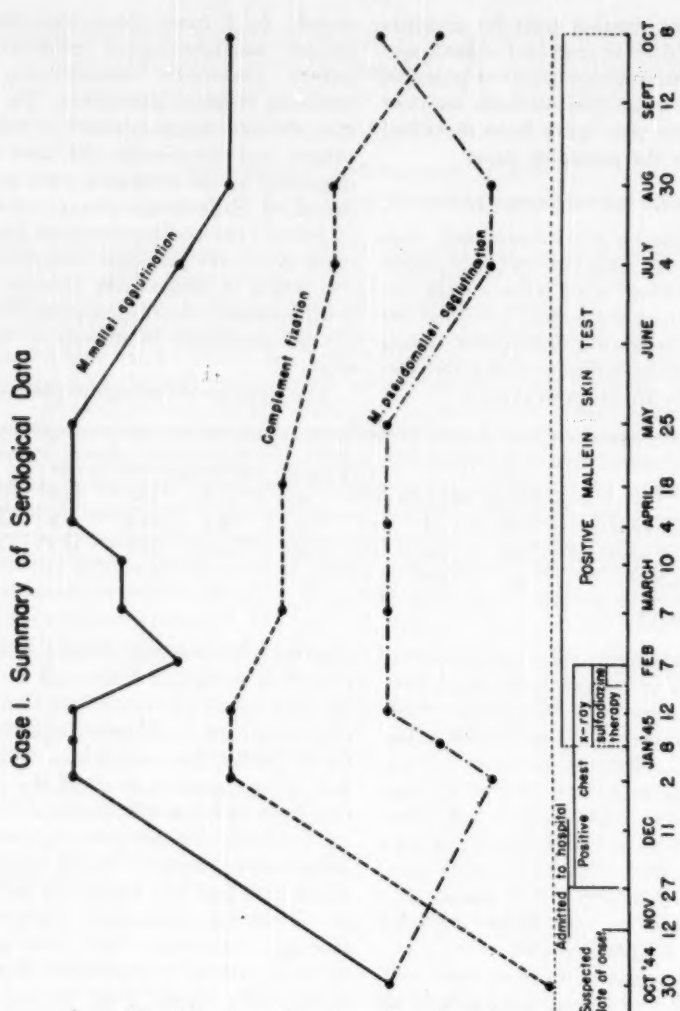

FIG. 1.—Pertinent serologic and clinical data in case I.

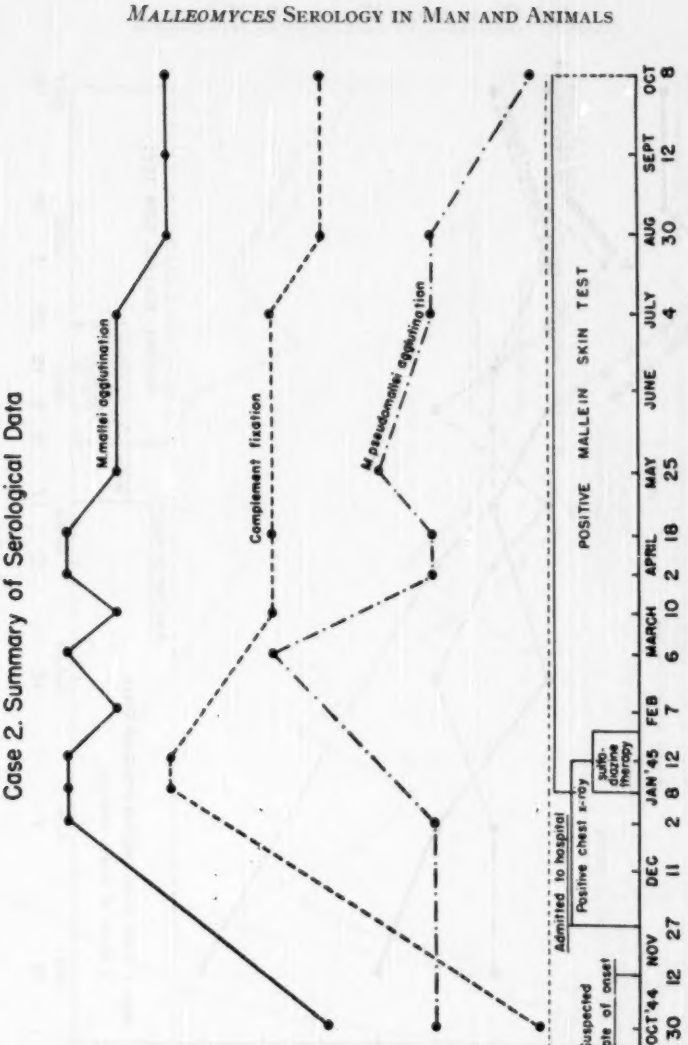

FIG. 2.—Pertinent serologic and clinical data in case 2.

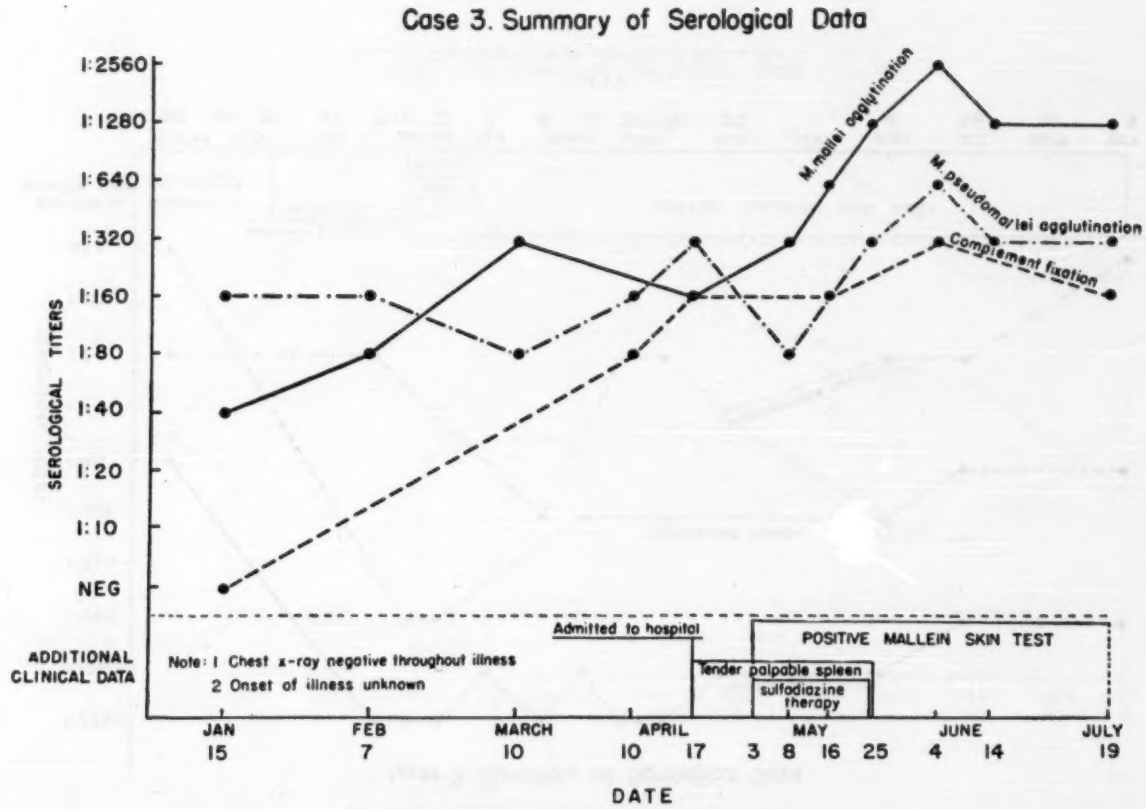

FIG. 3.—Pertinent serologic and clinical data in case 3.

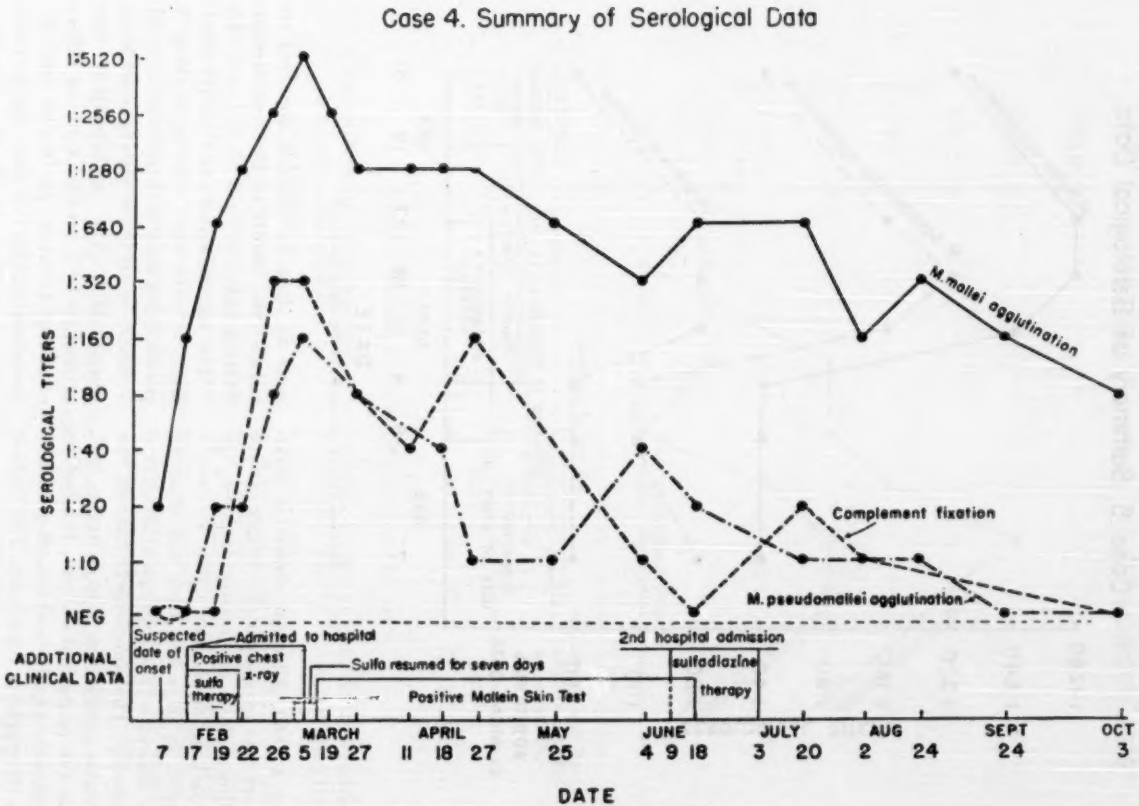

FIG. 4.—Pertinent serologic and clinical data in case 4.

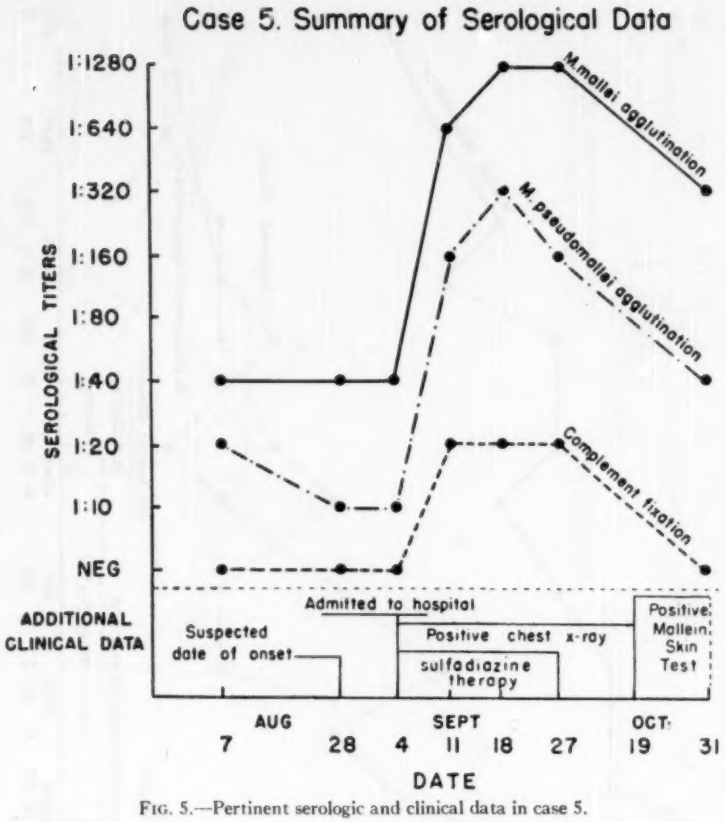

FIG. 5.—Pertinent serologic and clinical data in case 5.

and 1:2560. All cases showed a maximum titer over a variable length of time followed by a progressive decrease, approaching the normal value. In cases 1 and 2 an increased titer was observed for almost a year subsequent to clinical recovery. The complement fixation test became positive in 4 to 6 weeks, and, like the agglutination test, reached its peak titer rapidly and fell off gradually as the patients recovered. The highest complement fixation titer observed was 1:640.

All of the patients were given skin tests with a 1:10,000 dilution of commercial mallein. The results of the skin tests in these individuals appeared to parallel the results of the complement fixation test.

The agglutination and complement fixation tests were vital in making a positive diagnosis in these cases since all attempts to cultivate the organism were unsuccessful. The agglutination test proved to be a greater value in differentiating between *M. mallei* and *M. pseudomallei* infection than did the complement fixation test. In the agglutination test, there was a significantly higher titer with the homologous antigen, whereas the complement fixation test gave complete cross reactions.

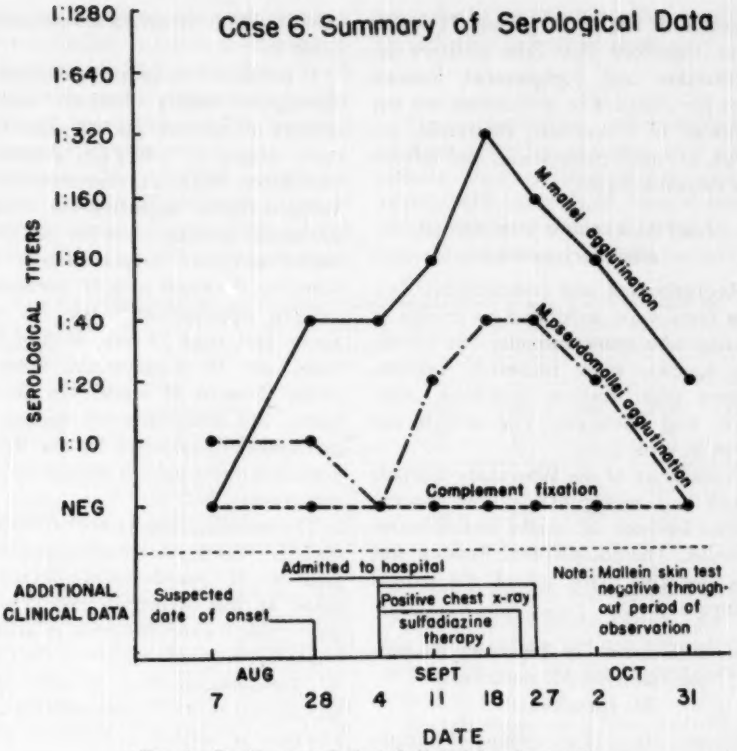

FIG. 6.—Pertinent serologic and clinical data in case 6.

Tests with serums from hospital cases other than glanders or melioidosis

In order to test the specificity of the agglutination and complement fixation tests, samples of serums from patients hospitalized with various diseases were tested. Serums were obtained from 38 cases of atypical pneumonia, 15 cases of anthrax, 5 cases of brucellosis, 7 cases of tularemia, and 3 cases of infectious mononucleosis.

A total of more than 150 blood samples were received from the 68 cases. The agglutination titers were found to be in approximately the same range as those observed in normal individuals, and the complement fixation tests were

TABLE 2.—M. mallei *and* M. pseudomallei *agglutination and complement fixation titers of normal animals.*

Animal	Number of animals tested	Agglutination titers		Complement fixation
		M. mallei	*M. pseudomallei*	
Rabbits	6	Neg.–1:20	Neg.	Negative
Guinea pigs	6	Neg.–1:20	Neg.–1:20	Negative
Ferrets	3	Neg.–1:40	Neg.–1:10	Negative
Monkeys	8	1:10–1:80	Neg.–1:20	Negative
Rats	4	Neg.–1:40	Neg.–1:20	Negative
Mice	6	Neg.	Neg.–1:10	Negative
Hamsters	6	Neg.–1:10	Neg.–1:20	Negative

negative in the entire group. It would seem, therefore, that false positive agglutination and complement fixation tests for glanders or melioidosis are not obtained in brucellosis, tularemia, anthrax, atypical pneumonia, and infectious mononucleosis.

Tests with serums from normal laboratory animals

Agglutination and complement fixation tests were performed on groups of normal laboratory animals. The following species were included: rabbits, guinea pigs, ferrets, monkeys, rats, mice, and hamsters. The results are given in table 2.

A majority of the laboratory animals tested had agglutinins in low concentration for both *M. mallei* and *M. pseudomallei*. The complement fixation test was negative with all of the animal serums tested.

Tests with serums of laboratory animals infected with M. mallei *and* M. pseudomallei

Guinea pigs.—Two groups of chronically infected guinea pigs were studied. One group was infected with *M. mallei* strain C4 and the other group was infected with *M. pseudomallei* strain W249. The characteristics of the disease in animals is reported elsewhere.[16] The results of the agglutination and complement fixation titrations are presented in table 3.

It is difficult to properly evaluate the serological results obtained with the serums of infected guinea pigs. In the early stages of infection, guinea pigs inoculated with *M. pseudomallei* developed higher agglutination titers for *M. mallei* antigen than for *M. pseudomallei* antigen. Several weeks later, however, the titers with *M. pseudomallei* antigen approximated the *M. mallei* titers, and after 75 days in 5 of 8 animals, the *M. pseudomallei* titers rose above those of *M. mallei*. On the other hand, *M. mallei*-infected guinea pigs produced higher titers for the homologous strain throughout the period of observation.

The complicating aspect of this study was the higher *M. mallei* agglutination titer in *M. pseudomallei*-infected animals. It was shown in the preceding paper that a close antigenic relationship exists between *M. mallei* strain C4 and *M. pseudomallei* strain W-294. Also, it was shown that *M. pseudomallei* agglutination is largely a function of the flagellar antigens. There is the possibility that flagellation was suppressed in the early stages of infection resulting predominantly in antibody response to somatic antigens. There is also the possibility that the "specific" *M. pseudomallei* antibodies were absorbed at the

TABLE 3.—*Agglutination and complement fixation titers with serums of infected guinea pigs.*

		25 days after inoculation			40 days after inoculation			75 days after inoculation		
		Agglutination		Complement Fixation	Agglutination		Complement Fixation	Agglutination		Complement Fixation
		M. mallei	*M. pseudomallei*		*M. mallei*	*M. pseudomallei*		*M. mallei*	*M. pseudomallei*	
Guinea Pigs (8) Infected with *M. mallei*	Range of titer	1:80–1:2560	1:20–1:160	0–1:160	1:160–1:2560	1:10–1:80	1:80–1:640	1:640–1:2560	1:20–1:160	1:160–1:1280
	Average titer	1:550	1:80	1:50	1:1040	1:39	1:290	1:1520	1:68	1:668
Guinea pigs (8) Infected with *M. pseudomallei*	Range of titer	1:320–1:1280	1:40–1:640	1:40–1:320	1:160–1:1280	1:80–1:640	1:40–1:640	1:80–1:640	1:160–1:1280	1:160–1:640
	Average titer	1:640	1:232	1:112	1:600	1:310	1:385	1:240	1:660	1:360

site of the infectious processes, leaving only nonspecific somatic antibodies in the circulation. The latter hypothesis may receive some support from the agglutinin absorption studies reported in the preceding paper.

Monkeys.—The normal agglutination and complement fixation titers of 6 monkeys were determined by a series of three titrations. The animals were then divided into two groups. One group was inoculated subcutaneously with graded doses of *M. pseudomallei* and the other group with graded doses of *M. mallei*. However, only one monkey in each group became infected.[15,16] These two animals were the only ones to show a significant change in the serological titers. The serological data obtained with each of these animals are given in table 4.

TABLE 4.—*Agglutination and complement fixation titers with serums of infected monkeys.*

Infected with	Serological test	"Normal" titer	Number of days following inoculation				
			10	20	25	32	47
M. mallei strain C4	*M. mallei* agglutination	80	320	1280	2560	2560	2560
	M. pseudomallei agglutination	20	80	40	160	1280	640
	Complement fixation	Negative	160	1280	1280	2560	640
M. pseudomallei strain W294	*M. mallei* agglutination	20	80	160	640	1280	640
	M. pseudomallei agglutination	20	80	160	1280	1280	1280
	Complement fixation	Negative	Negative	320	320	640	640

The serum from the *M. mallei* infected-animal gave a high agglutination titer with homologous antigen and a low titer with *M. pseudomallei* antigen. The *M. pseudomallei*-infected animal produced agglutinins in high titer for both *M. mallei* and *M. pseudomallei*, with the latter being somewhat higher. Complement-fixing antibodies were produced to high titer in both animals.

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SUMMARY

The agglutination and complement fixation tests for glanders and melioiodosis were performed on approximately 400 samples of normal human serums. Ninety-seven to 98% of the normal serums had agglutinins for both *M. mallei* and *M. pseudomallei*, ranging in titer from 1:10 to 1:320. The complement fixation test gave negative results with all of the normal serums tested.

Six laboratory infections with *M. mallei* presented an opportunity to evaluate the serological tests as a means for diagnosing the disease in man. Both the agglutination and complement fixation tests were valuable in establishing the diagnosis. It was shown that a definite rise in the agglutination titer occurred 2 to 3 weeks subsequent to infection, followed by a progressive rise to 1:1280 and 1:2560. A high titer was maintained for from 3 to 12 months after infection, following by a steady decrease approaching the normal value. The complement fixation test became positive in about 4 to 6 weeks and, like the agglutination test, reached its peak rapidly and fell off gradually as the patient recovered. The highest complement fixation titer observed was 1:640.

A total of more than 150 samples of

serums from patients hospitalized with brucellosis, tularemia, anthrax, atypical pneumonia, and infectious mononucleosis were tested by the agglutination and complement fixation tests for false positive reactions. The agglutination titers were all within the range observed in normal individuals, and the complement fixation tests were negative in the entire group.

It was found that normal rabbits, ferrets, guinea pigs, monkeys, rats, mice, and hamsters have agglutinins in low titers for both *M. mallei* and *M. pseudomallei*.

A group of chronically infected guinea pigs was followed periodically by serum agglutination and complement fixation titrations. The *M. pseudomallei*-infected guinea pigs in the early stages of the disease gave higher agglutination titers with *M. mallei* antigen than with homologous antigen. Several weeks later, however, the *M. pseudomallei* titers approximated the *M. mallei* titers, and after 75 days the *M. pseudomallei* titers rose above the *M. mallei* titers. The *M. mallei*-infected guinea pigs, on the other hand, produced higher agglutinin titers for *M. mallei* than for *M. pseudomallei* throughout the course of the disease.

Two monkeys, one infected with *M. mallei* and the other infected with *M. pseudomallei*, gave agglutination and complement fixation titers similar to those obtained in human cases. The *M. mallei*-infected monkey developed agglutinins for *M. mallei* to high titer and for *M. pseudomallei* in low titer. The *M. pseudomallei*-infected animal produced agglutinins in high titer for both *M. pseudomallei* and *M. mallei*, with the former somewhat higher. Complement-fixing antibodies were produced in both animals to a titer of 1:640.

CONCLUSIONS

1. The complement fixation test appeared to be specific for *Malleomyces* infection but did not differentiate between glanders and melioidosis.

2. The agglutination test was more sensitive than the complement fixation test, and practically all normal serums gave agglutination in low dilution. If the normal agglutinin titers have been previously determined, an early progressive rise in titer may be diagnostic of the disease before the complement fixation test becomes positive.

3. The agglutination and complement fixation tests are excellent diagnostic aids for glanders and melioidosis in man as well as in animals.

A CORRELATION OF ANTIGENIC CHARACTERISTICS AMONG CERTAIN BACTERIA OF THE LACTOBACILLUS GROUP

FRANK J. ORLAND

From the Department of Bacteriology and Parasitology and the Walter G. Zoller Memorial Dental Clinic, The University of Chicago, Chicago 37, Illinois

Antigenic characteristics of the bacteria belonging to the *Lactobacillus* group have been studied by a considerable number of investigators during the past three decades, but the accrued knowledge regarding lactobacillus antigenicity is still difficult to interpret and is most certainly incomplete. A comprehensive review of the literature pertaining to antigenic relationships among lactobacilli as a whole apparently has never been prepared despite the controversial though highly interesting field of investigation that it has been. The various serological studies reported to date can be divided into several categories on the basis of the particular problems investigated.

A. The least involved problem concerned itself with the relationship of Döderlein's vaginal bacillus to *Lactobacillus acidophilus* Moro of the intestinal tract. The observation of Jötten (1922) that these two lactobacillus forms were serologically the same was substantiated by the later report of Thomas (1928). Lash and Kaplan (1926), though they found antigenic differences among strains of Döderlein's bacillus, thought it likely, nevertheless, that some close relationship existed among them.

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B. At one time considerable interest was displayed as to the relationship of *Lactobacillus bifidus* and *L. acidophilus* since both are found in the intestinal tract of infants and apparently depend upon the diet as to which form predominates. First, Cruickshank and Berry (1924) and then Cruickshank (1925) stated that these intestinal forms were closely related antigenically although their strains of *B. bifidus* were not all uniform in this respect. Despite the report of Boventer (1938) that *B. acidophilus* and *B. bifidus* could be differentiated serologically, Weiss and Rettger (1934) in a comprehensive study declared that although there were great variations among different strains of each of these two groups, *L. bifidus* was considered to be a variant of *L. acidophilus*.

C. As a consequence of the therapeutic attempt to implant lactobacilli in the intestinal tract, there arose the controversy regarding the relationship of *Lactobacillus bulgaricus* and *L. acidophilus*. Metchnikoff had tried to implant *L. bulgaricus*, but others thought *L. acidophilus*, being a natural inhabitant of the intestine, was more suitable. McIntosh et al (1924) provided some serological evidence that certain strains of *B. bulgaricus* were different from both their types I and II *L. acidophilus odontolyticus*. Despite this observation, Kulp and Rettger (1924) in their comprehensive study found no greater difference between *B. bulgaricus* and *B. acidolphilus* than could be expected between members of the same species, and hence they concluded that the former

was merely a variant of the latter. Further evidence by Upton and Kopeloff (1932) strengthened this view; these investigators showed rough forms of *L. bulgaricus* and *L. acidophilus* were antigenically the same. Similarly, smooth forms of these two species were also found to be the same. But conversely, the R and S forms of either species were antigenically dissimilar. Markoff (1943) also indicated *T. bulgaricum* was related to *T. intestinale*,* but Thomas (1928) had provided serological data to show that *L. bulgaricus* differed from both Döderlein's bacillus and *L. acidophilus*. The preponderance of evidence, however, seems to indicate that antigenically the two forms are at least closely related.

D. Until very recent times, there persisted the highly controversial problem concerning the relationship of oral and intestinal strains of lactobacilli. McIntosh et al (1924) showed a close serological relation among members of the "acidophilus group" although such strains isolated from milk and human sources seemed to have a closer relation to their stock oral strains than to those obtained from the intestines of monkeys and rats. In his serological study of "Milchsaürebakterien," Hilgers (1924) provided some evidence that at least some strains of *B. necrodentalis* (as oral forms had been termed) were quite closely related to *B. acidophilus* of the intestines. Ross et al (1927) found cross agglutination between the two forms but in low titer. Similarly, Morishita (1928 and 1929) observed no cross agglutination except in low dilution of the serum. Rosebury et al (1929) provided substantial evidence that strains of both groups showed variability and cross agglutination and therefore were thought to be the same. Howitt (1930) also amassed data which indicated the two groups were similar. Hunt and Rettger (1930), using some of Morishita's oral strains, found no cross reaction between these and a strain of *L. acidophilus* nor between two strains of intestinal *L. acidophilus*. Upton and Kopeloff (1932) in their work on dissociation concluded the oral strains were closely related to intestinal forms. Enright et al (1932) found no difference within their group I lactobacilli regardless of oral or intestinal origin. Tests conducted with a few antiserums in addition to other observations led Anderson and Rettger (1937) to regard their oral strains as a group distinct from intestinal *L. acidophilus*. However, Warner and Arnold (1941) stated their dental strains showed homogeneity but as a group appeared not to be distinct from *L. acidophilus*. By the use of carbohydrate precipitation methods, Harrison and Opal (1944) showed intestinal and oral strains from the same individual were apparently identical. It is not exactly clear why diametrically opposite views arose regarding the relationship of oral and intestinal lactobacilli when most studies provided only rather inconclusive data on which conclusions were based. Nevertheless, the preponderance of evidence amassed by many laboratories seems to substantiate the ideas expressed by Hemmens (1948) in her review of this topic that at present the weight of evidence appears to favor the essential identity of *L. acidophilus* (Moro) with the lactobacilli inhabiting the oral cavity. As a possible explanation of the reported discrepancies, it may be that the particular strains tested, though considered "characteristic" of their particular environment, were actually only variants of the same fundamental *Lactobacillus* form and on subculture in identical environments

* *T. intestinale* refers to *L. acidophilus*. In all other instances as well, the original designations for the various lactobacilli have been used.

they tended to lose their differences and become indistinguishable.

E. Antigenic studies confined exclusively to the oral strains have been more limited in number, but some are of considerable significance. While Sierakowski and Zajdel (1924) observed only uniformity among their oral strains and Warner and Arnold (1941) concluded their dental strains showed homogeneity among themselves, Howitt (1930), on the basis of her comprehensive study, suggested that oral lactobacilli belong to separate groups rather than all to one and the same group. The report of Enright and associates (1932) as well as the earlier work of McIntosh et al (1922) provided some serological evidence for this thought by differentiating between two groups or types of oral lactobacilli. Then Harrison et al (1939) showed there existed at least four serological types in one group of oral strains while another group consisted of heterogenous types. Later Harrison (1942a) reported that by their fermentation reactions his oral lactobacilli fell into two groups essentially identical with his two large serological groupings. On the basis of serological studies, Canby and Berniers (1942) concluded that the antigenic structure of lactobacilli must be extremely complex and the number of antigenic components very great. Associated with these thoughts, Harrison contributed the concept of overlapping antigens among oral strains and this concept apparently induced Williams (1948a, 1948b, 1949) to work out different antigenic components in a limited number of oral lactobacilli. This successful attempt at antigenic analysis of these microorganisms is considered highly significant since such an approach may help to clarify the interrelationship of not only oral strains but of lactobacilli found in other environments as well.

F. A few additional observations on other aspects of lactobacillus antigenicity aside from those discussed thus far are the following. Yamada (1923) reported he could separate different strains of *B. acidophilus*, while McIntosh et al (1924) noted strains isolated from the intestine of monkeys and rats were not related to their human oral strains and to those from milk and other human sources. Hishikari (1924) reported that B. *bulgaricus* and *B. bifidus* reacted quite differently in serological tests. Hunt and Rettger (1930), in their broad study of lactobacilli comprising various species isolated from different sources, observed certain gross relationships but in the absence of antigenic analyses, the reactions as a whole seemed variable and not very significant. The antigenic and other characteristics of the strains isolated from dry wine by Douglas and Cruess (1936) seemed to be somewhat different from other well known lactobacilli. Two other strains which were demonstrated to be serologically identical by Biocca and Reitano (1943) and by Biocca and Seppilli (1947) and allegedly caused two fatal cardiac involvements seemed rather unique in their pathogenic capabilities. In both of these instances it is problematical, however, whether the respective strains mentioned should be designated as a new species in the one case and a new variety in the other without further intensive study.

The foregoing evaluation of the literature concerning the antigenic interrelationship of various lactobacilli was considered essential for the interpretation of findings to be presented in this report.

The purpose of the present study was to extend the antigenic analyses of lactobacilli so as to include representatives of all available species as well as strains isolated from a wide variety of sources.

An attempt was then made to correlate the determined antigenic components with other characteristics exhibited by lactobacilli. It was also deemed important to study the stability of the more prevalent antigens under various environmental conditions.

MATERIALS AND METHODS

Stock cultures.—The nature of this investigation necessitated the collection of lactobacilli comprising most species listed in Bergey's Manual as well as other strains isolated from a variety of sources. These cultures were obtained either as donations from other laboratories or were isolated in this laboratory. The following individuals and groups contributed to the collection of lactobacilli: F. M. Clark, W. E. Gaunt, M. E. Hanke, R. W. Harrison, W. C. Haynes, K. E. Hite, M. Muedeking, M. V. Novak, C. S. Pederson, L. F. Rettger, W. H. Riesen, E. E. Snell, R. P. Tittsler, M. Wagner, N. B. Williams, H. B. Woodruff, the American Type Culture Collection, and the National Collection of Type Cultures. The strains collected were isolated from various dairy products such as milk and cheeses, from fermented vegetable foodstuffs as sauerkraut and cucumbers, from the oral cavity of man, rats, and hamsters, and from the vaginal and intestinal tract of man and rats.

Stock cultures were obtained by single colony isolations from agar plates composed principally of tomato juice and yeast extract. This medium is selective for such aciduric microorganisms as lactobacilli and has been used previously in this laboratory (Orland, 1946; Williams, 1948a). The 250 cultures in the collection were transferred to fresh tomato juice yeast extract (TJY) broth at monthly intervals, usually under a hood to minimize contamination. Since there is evidence in this laboratory that some lactobacilli may undergo changes while being transferred through ordinary medium (Harrison, 1942b; Coolidge et al, 1949), certian representative strains were frozen and stored in a carbon dioxide dry ice chamber.

Vaccine preparation.—For the production of serum antibodies in rabbits, vaccines were prepared from certain strains of lactobacilli. Cells were grown in 50 to 400 ml of TJY broth for 3 to 4 days at 37 C, depending on the growth ability of the strains concerned. The cells were aggregated by centrifugation, being washed three successive times in sterile 0.85% saline. Suspensions for immunization were adjusted to a turbidity reading of 20 on a Cenco photelometer scale using the green filter so there would be approximately 2.5×10^9 cells per ml of saline. The vaccine, placed in glass-stoppered bottles, was heated to 65 C for one hour, tested for viable cells on TJY agar plates, and then stored in the refrigerator for rabbit immunization.

Immunizations.—Young rabbits weighing between 2 and 4 kg were used for antibody production. An initial venipuncture provided normal serum for determination of the natural antibody titer against the strain of lactobacillus to be used for immunization. There was, however, rarely a measurable normal titer present in the rabbits used. Usually two or more animals were immunized with the same vaccine by a short term of injections as followed by Williams (1948a) in his work. This consisted of a series of 5 intravenous injections of 0.2 ml doses of the vaccine on alternate days. Titer determinations were made on serum obtained 5 days after the last injection of the series. If agglutination could not be observed at the 1:1280 dilution level of the serum, the animal was given 2 or 5 additional injections, and this almost always carried the titer to at least 1:1280. It was observed that those strains of lactobacilli not eliciting an adequate titer in three successive series of injections seldom showed one even with a considerable number of additional injections. Rabbits whose serums exhibited titers over 10^{-3} were bled either by auricular venipuncture or by cardiopuncture. The serums were preserved with 0.2% phenol and stored in the refrigerator.

Agglutination tests.—On the basis of early studies with lactobacilli it became evident that although various serological methods were employable, agglutination of the whole bacterial cell was the simplest and seemed adequate enough for merely surveying the antigenic nature of bacteria belonging to the lactobacillus group. The antigens used in these tests were prepared by the following procedure. Lactobacillus cells were allowed to propagate in quantities of TJY broth. After centrifugal aggregation and triple washing in large amounts of sterile distilled water to remove broth ingredients, the cells were resuspended in sterile distilled water in order to minimize autoagglutination, as was done by Weiss and Rettger (1934) and Williams (1948a). Following vacuum filtration through cotton, the suspension was stored in the refrigerator with 0.2% phenol as preservative.

A screening test consisting of rapid agglutination on a slide observable macroscopically was used in many instances to detect certain antigens preliminarily. However, the usual tube agglutination test always provided the conclusive information regarding lactobacilli studied in any detail. In performing this simple slide test, a heavy

water suspension of bacterial cells with a density of 5 on the photelometer scale, which was equivalent to approximately 5.8×10^9 cells per ml, was used as the antigen. The antiserum was diluted to 1/20 of its highest titer as determined by the homologous antigen in a tube agglutination test. With a capillary pipette one drop of cell suspension was added to a drop of the diluted serum in the concavity of a hollow ground slide. The two substances were intermixed by means of a short wood stick and an electric vibrator. With oblique illumination, results were read 5 to 15 minutes later and recorded as positive or negative on the basis of comparison with a negative control (wherein saline replaced the antiserum) and a positive control (consisting of the cell suspension and the homologous antiserum). In general, it was found that a negative slide agglutination almost invariably resulted in a negative or very low-titered tube agglutination. On the other hand, even with the standardized procedure used, a positive slide agglutination either could be substantiated by the regular tube agglutination test showing a high titer over 1:1280 or would result in a lower titer considered inadequate for demonstration of a major antigen. Thus every slide test showing some trace of agglutination was followed by the standard tube agglutination test for verification.

In performing the tube agglutination test, the serum to be tested was diluted with a compensatory 1.7% solution of sodium chloride so that the final concentration approximated 0.85% since 0.2 ml of serum was mixed with an equal volume of aqueous suspended cells. This cell suspension was standardized at 9.5×10^9 cells per ml or a reading of 45 on the photelometer scale. Final dilutions ranged between 1:20 and 1:5120, having been prepared by the double dilution method. Decimal dilutions up to and beyond 10^{-3} were also used. Dilution procedures were devised so that cells of many strains could be tested with an antiserum at one time. The tests were read following two hours' incubation in a 37 C water bath and storage in the refrigerator for 12 to 18 hours thereafter. The titer was recorded as the highest dilution at which there occurred a maximal 4 plus or at least a 3 plus agglutination; anything less was considered inadequate. Any sign of autoagglutination in the control tubes made it mandatory to discard the test.

Agglutinin absorption.—In order to display the multiplicity of antigenic components which some strains of lactobacilli possessed, it was necessary to absorb specific agglutinins from the antiserums to obtain so-called monospecific or univalent preparations. These antiserums were then utilizable for the detection of corresponding specific antigenic components among the various strains of lactobacilli. Another reason for use of the absorption procedure was to verify by reciprocal agglutinin absorption the individuality of the antigenic components found or, conversely, to provide evidence for the antigenic similarity of two strains of lactobacilli. In the procedure termed mirror absorption, just mentioned, antiserum of one strain was absorbed by the cells of another strain and reciprocally the antiserum of the second strain was then absorbed by cells of the first.

To perform these agglutinin absorption tests, large quantities of bacterial cells were required. These were grown in flasks of TJY broth, washed and sedimented by centrifugation, and packed into conical, calibrated centrifuge tubes to the 0.5 ml mark. This quantity of cells was mixed with 5 ml of undiluted serum to be absorbed. Despite larger or smaller quantities of serum used, the same approximate ratio of cells to serum was maintained. The mixture was placed in a rubber-stoppered tube and in a 37 C water bath. The latter was equipped with a motor-driven shaking apparatus and an automatic timer to permit shaking for exactly two hours at one time. After absorption, the absorbing antigen was sedimented by centrifugation and the serum was removed with a capillary pipette. This process of absorption was repeated, and in most instances three consecutive absorptions seemed to have removed all evidence of the agglutinins concerned although in some instances only one absorption was required and in other instances as many as six absorptions were necessary.* The final absorbed serum after preliminary testing was tubed, labeled, and stored for future use.

Antigen stability tests.—Interest was developed beyond the identification of innumerable antigenic components occurring in lactobacilli. It seemed relevant to learn something of the more intrinsic traits of the antigens themselves. What environmental conditions, for example, could the bacterial cells undergo and still possess demonstrable agglutinogens? Severe physical changes in the environment of the washed cells included boiling for two hours, steaming at 15 pounds pressure for 15 minutes, and freezing. Suspensions of cells were also treated with 50% and absolute alcohol, as well as with $N/16$ and $N/1$ hydrochloric acid, and $N/10$ sodium hydroxide for differ-

* On the basis of their agglutinin absorptions, Upton and Kopeloff (1932) concluded only one absorption with lactobacilli was necessary since serums absorbed once with homologous antigen exhibited a titer of only 5% or less of the final titer possessed by the unabsorbed serum.

ent periods of time and at different temperatures. Another approach to the effect of environment on the antigen concerned was to grow the particular culture in medium which had been modified by the addition of sublethal quantities of growth-influencing agents. Such substances were phenol, sodium fluoride, sodium chloride, normal rabbit serum, specific antiserum, and penicillin. In these studies it was necessary to carry the strains through a large number of serial transfers in TJY broth containing these substances but in quantities below a bactericidal level. At intervals and at termination of the serial transfers, grown cells were tested for the presence of the antigenic components as well as for the characteristic fermentation pattern which the parent cells originally exhibited.

Fermentation tests.—Lactobacilli have been segregated into subgeneric groups to a considerable extent on the basis of the sugars they fermented. Hence it seemed quite important to consider the carbohydrate fermentation patterns in correlation with the antigenic traits of these bacteria. One half percent of the carbohydrate to be tested was added to culture tubes of veal infusion broth containing 0.0016% bromcresol purple as an indicator of acid productivity. The broth was steam sterilized at 10 to 11 pounds pressure for 20 minutes, which was sufficient for sterilization but apparently did not cause disintegration of the sugars, at least as far as could be determined by collateral tests wherein the more heat labile sugars had been rendered sterile by filtration instead of by steam. Following inoculation, the results of the bacterial action as compared to uninoculated control tubes were read at 1-, 2-, 3-, 7-, and 14-day intervals. Besides litmus milk, the following carbohydrates were used, although not all were tested by every lactobacillus strain studied antigenically. The monosaccharides used included the pentoses *l*-rhamnose, *l*-sorbose, *d*-xylose, *d*-ribose, and *l*-arabinose, while the hexoses were *d*-glucose, *d*-fructose, *d*-galactose, and *d*-mannose. Disaccharides tested were lactose, sucrose, maltose, trehalose, melibiose, and cellobiose. Polysaccharides used consisted of raffinose, melezitose, starch, inulin, dextrin, and glycogen. Alcohols included glycerol, erythritol, adonitol, mannitol, sorbitol, dulcitol, and inositol. Salicin, aesculin, and α-methyl glucoside composed the group of glucosides tested.

Acidimetric titrations.—Since one of the salient characteristics of the lactobacillus group is the production of relatively large quantities of acid, it was deemed appropriate to quantitatively determine the titrable acid produced, as a potential means of strain differentiation. The lactobacilli to be tested were grown for 10 days at 37 C in culture tubes containing exactly 10 ml of a partly synthetic medium containing 1% glucose. The composition was as follows: peptone, 1 *g*, cystine; 0.1 *g*; yeast extract, 2 g; glucose, 2 g. The minerals were the same as those used by Speakman (1923): K_2HPO_4, 100 mg; KH_2PO_4, 100 mg; $MgSO_4 \cdot 7H_2O$, 40 mg; NaCl, 2 mg; $FeSO_4 \cdot 7H_2O$ 2 mg; $MnSO_4 \cdot 4H_2O$, 2 mg; and distilled water quantum sufficit 200 ml. Preliminary experiments verified the general observation that most strains of lactobacilli will not utilize to any appreciable extent the acid produced by carbohydrate dissimilation during this 10-day period. Actually, an almost equal amount of acid could be titrated at about 6 to 8 days. However, the 10-day period was selected in order to be sure of maximal acid productivity and also because Pederson (1938) utilized 10 days in his extensive acid titrations of lactobacilli. Subsequent to the incubation period, the amount of growth and the pH were recorded while the titration results were expressed as ml of 0.1 *N* titrable acid.

Optimal temperature determinations.—Each strain was maintained at: 10, 24, 30–32, 37, 45, and 50 C. Tests were repeated a number of times for verification, especially in instances where growth seemed equally abundant over a range of temperatures for the same period of incubation.

Growth on blood agar.—In preliminary tests it was observed that certain lactobacillus colonies would grow on the surface of blood agar with a circumventing halo having a slight green or bleached appearance. Other colonies would show no zonal reaction, and still others would not even grow. To test representative strains of lactobacilli for these characteristics 10% defibrinated sheep blood agar was used. Both surface and deep colonies were observed at intervals up to 14 days' incubation at 37 C.

RESULTS

Antigenic studies.—From among the 252 cultures studied, only 33 were found to exhibit one or more of the major antigens for which they were tested. Of this number, 30 strains showed the presence of a common antigen designated as *F* (since this was the 6th distinct antigen found among loctobacilli. It has not necessarily any relationship to Forssman's heterophile antigen sometimes referred to as the F antigen). An antigen termed *G* was observed in two other strains, and in another strain an

TABLE 1.—*Results of agglutination reactions.*

Lactobacilli manifesting major antigens			Antiserums with agglutinins for only one major antigen								
Laboratory number	Species designation	Strain number	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*
L-301	*L. casei*	7469	−	−	−	−	−	+	−	−	−
L-304	*L. plantarum*	10012	−	−	−	−	−	−	+	−	−
L-305	*L. casei*	BC-1	−	−	−	−	−	+	−	−	−
L-309	*L. bulgaricus*	7995	−	−	−	−	−	+	−	−	−
L-310	*L. bulgaricus*	7995	−	−	−	−	−	+	−	−	−
L-311	*L. helveticus*	L43	−	−	−	−	−	+	−	−	−
L-313	*L. delbrueckii*	5R9573	−	−	−	−	−	+	−	−	−
L-320	*L. casei*	7469	−	−	−	−	−	+	−	−	−
L-323	*L. casei*	393	−	−	−	−	−	−	−	+	−
L-324	*L. casei*	7469	−	−	−	−	−	+	−	−	−
L-326	*L. casei*	(LD-S) 9595	−	−	−	−	−	+	−	−	−
L-330	*L. casei*	1438	−	−	−	−	−	+	−	−	−
L-336	*L. delbrueckii*	1358	−	−	−	−	−	+	−	−	−
L-339	*L. casei*	ZBL	−	−	−	−	−	+	−	−	−
L-340	*L. casei*	6557	−	−	−	−	−	+	−	−	−
L-432	*L. casei*	7469	−	−	−	−	−	+	−	−	−
L-352	*L. casei*	7469	−	−	−	−	−	+	−	−	−
L-354	*L. casei*	LD-5	−	−	−	−	−	+	−	−	−
L-356	*L. casei*	(V300) 7469	−	−	−	−	−	+	−	−	−
L-369	*L. casei*	V349	−	−	−	−	−	+	−	−	−
L-371	*L. plantarum*	V731	−	−	−	−	−	−	+	−	−
L-373	*L. acidophilus*	(milk)	−	−	−	−	−	+	−	−	−
L-374	*L. casei*	7469	−	−	−	−	−	+	−	−	−
L-377	*L. casei*	7469	−	−	−	−	−	+	−	−	−
L-378	*L. casei*	4796	−	−	−	−	−	+	−	−	−
L-379	*L. casei*	Ldg	−	−	−	−	−	+	−	−	−
L-380	*L. delbrueckii*	Ld4913	−	−	−	−	−	+	−	−	−
L-381	*L. delbrueckii*	7R5676	−	−	−	−	−	+	−	−	−
L-390	*L. casei*	(*a*) 7469	−	−	−	−	−	+	−	−	−
L-391	*L. helveticus*	B-442	−	−	−	−	−	+	−	−	−
L-392	*L. delbrueckii*	B-445	−	−	−	−	−	+	−	−	−
L-641	(oral strain)		−	−	−	−	−	+	−	−	+
L-652	(oral strain)		−	−	−	−	−	+	−	−	−

Major antigens were considered demonstrable by agglutination titers over 10^{-3} (almost always at 1:1280 or 1:2560). Such reactions are indicated by plus (+), whereas a negative (−) sign indicates no titer or a very low titer.

antigen called *H* was found. Still another antigen designated as *I* was demonstrated to be an antigenic component of a strain also possessing an *F* antigen. Additionally, a number of ostensible antigens or antigenic components were encountered in other lactobacilli, but these could not be demonstrated consistently by the methods of analysis employed. Hence they were considered to require further study before being reported upon. Another group of strains expressed themselves on a lower level of antigenicity, as indicated by low agglutination titers in both homologous and heterologous antiserums. These reactions in all likelihood should be considered as expressions of minor antigens; further study of them is also indicated. Thus presentable findings are limited to those concerned with antigens which consistently manifested themselves in relatively high agglutination titers of at least 10^{-3}.

In table 1 results are presented of homologous and heterologous agglutination tests. For each distinct major antigen, monospecific serum was prepared whenever necessary by the absorption of all agglutinins except the one type desired. The monospecific agglutinins are designated by the lettered antigens found in lactobacilli against which they react.

A salient finding among the strains listed in table 1 was that a considerable number of *Lactobacillus casei* strains were agglutinated in anti-*F* serums to a titer of at least 1:1280. This was interpreted to mean these cultures possessed a common major antigen which logically should be designated as *F*. It seemed

notable that *L. casei* cultures having the same A.T.C.C. strain number 7469, although obtained from different laboratories where they undoubtedly were maintained under different environmental conditions for long periods of time, still manifested the presence of the same antigen. Nine other cultures of *L. casei* with different strain numbers also exhibited an *F* antigen, but in 8 other *L. casei*-designated cultures, no such antigen could be demonstrated. On the other hand, a not inconsiderable number of lactobacilli other than *L. casei* did manifest *F* antigens. This group included 2 strains designated as *Lactobacillus helveticus*, 2 as *Lactobacillus bulgaricus*, 5 as *Lactobacillus delbruekii*, 1 as *L. acidophilus*, and a few strains isolated from the human oral cavity.

A culture labeled *Lactobacillus plantarum* 10012 was found to show the presence of none of the earlier established antigens but did exhibit another antigen termed *G*. Some time later, another strain of *L. plantarum* showed the presence of this same antigen and upon inquiry it was learned the second strain also carried the same A.T.C.C. number of 10012. It was also observed that one of the *L. casei* strains (A.T.C.C. #393) not exhibiting an *F* antigen and originally isolated from cheese elicited a major antigen sequentially designated as *H*. The antigen called *I* was found in the following manner: It was tentatively determined, by cell agglutination to a titer of 1:1280 in an anti-*F* serum, that the oral strain L-641 thereby possessed an *F* antigen. By agglutinin absorption of the L-641 antiserum with L-301 cells which had an *F* antigen, it was found that L-641 cells were still agglutinated by their homologous absorbed serum to almost the same titer. This led to the postulation that L-641 must contain another antigenic component in addition to *F*; this, then was designated as *I*. Conversely, however, absorption of L-301 antiserum by L-641 cells apparently removed all the *F* agglutinins and hence L-301 cells were no longer agglutinated in their homologous absorbed serum. Thus, with the absence of any of the earlier designated antigens in both L-641 and L-301 cells, it was postulated that L-641 cells must contain at least the two major antigens *F* and *I*, whereas L-301 cells contain the antigen *F* but not *I*, and may or may not contain any other antigen not yet demonstrated in L-641 cells.

Other cultures of lactobacilli with an *F* antigen did not show the presence of any additional major antigens demonstrable to date. This also seemed to be true of the cultures exhibiting the antigens *G* and *H*, respectively.

To establish unequivocally that all of the major antigens demonstrated thus far in different strains of the *Lactobacillus* genus are really separate entities, it was imperative to perform reciprocal agglutinin absorption tests among various representative strains manifesting one or more of these major antigens. Besides testing those cultures found to have an *F*, *G*, *H*, or *I* antigen present, cultures with the antigens *A*, *B*, *C*, *D*, or *E*, as designated earlier by N. B. Williams, were also included in order to make the reciprocal absorption testing comprehensive in scope. Table 2 depicts in simplified form the results of these tests among representative strains of lactobacilli and monospecific antiserums. As exemplification, cells with an *F* antigen (strain L-301 in this instance) were used in absorbing *f* agglutinins from the appropriate antiserum (developed against strain L-652). In subsequent agglutination tests this absorbed serum failed to agglutinate cells having an *F* antigen, thus indicating the complete loss of *F* agglutinins. On

TABLE 2.—*Results of reciprocal agglutinin absorption.**

Major antigens found in representative strains of lactobacilli	Agglutinins present in monospecific antiserums								
	a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*
A						*f*	*g*	*h*	*i*
AB						*f*	*g*	*h*	*i*
C						*f*	*g*	*h*	*i*
BD						*f*	*g*	*h*	*i*
E						*f*	*g*	*h*	*i*
F	*a*	*b*	*c*	*d*	*e*	0	*g*	*h*	*i*
G	*a*	*b*	*c*	*d*	*e*	*f*	0	*h*	*i*
H	*a*	*b*	*c*	*d*	*e*	*f*	*g*	0	*i*
FI	*a*	*b*	*c*	*d*	*e*	0	*g*	*h*	0

* In each instance monospecific antiserum was mixed with heterologous or homologous lactobacilli possessing one or more major antigens. The agglutinins which remained unabsorbed after such a procedure are indicated by small letters in the body of the table. Where all known agglutinins were absorbed from an antiserum, the designation 0 was used. Only 56 different absorptions were performed instead of the total number of 81, since the earlier antigens (*A* through *E*) already had been established as separate entities by Williams (1948a, 1948b, 1949).

the other hand, when L-304 antiserum containing *g* agglutinins was incubated with *F* cells (L-301), these agglutinins were not absorbed as shown by the unaltered agglutination of *G* (L-304) cells by the absorbed serum. This same testing procedure was carried out with each of the different monospecific serums absorbed by cells containing the different major antigens under study. Taking the results of the 56 absorptions as evidence, it can be stated with considerable cer-

TABLE 3.—*Similarity of fermentative and other characteristics of strains exhibiting an* F *antigen.*

Laboratory number	Species designation	Strain number	Carbohydrates*																Titrable acidity in ml of *N*/10 NaOH	Average optimal temperature
			Arabinose	Rhamnose	Sorbose	Glucose	Lactose	Sucrose	Trehalose	Melibiose	Cellobiose	Raffinose	Melezitose	Inulin	Dextrin	Mannitol	Sorbitol	Dulcitol	Salicin	
L-301	*L. casei*	7469	0	+	+	+	+	0	+	0	+	0	+	0	0	+	+	0	+	9.3	37 C
L-320	*L. casei*	7469	0	+	+	+	+	0	+	0	+	0	+	0	0	+	+	0	+	9.6	37
L-324	*L. casei*	7469	0	+	+	+	+	0	+	0	+	0	+	+	0	+	+	0	+	9.5	37
L-342	*L. casei*	7469	0	+	+	+	+	±	+	0	+	0	+	0	0	+	+	0	+	9.8	37
L-352	*L. casei*	7469	0	+	+	+	+	0	+	0	+	0	+	0	0	+	+	0	+	8.6	37
L-374	*L. casei*	7469	0	+	+	+	+	+	+	0	+	0	+	0	0	+	+	0	+	8.8	37–45
L-305	*L. casei*	BC-1	0	+	+	+	+	0	+	0	+	0	+	0	0	+	+	0	+	9.6	37
L-390	*L. casei*	c	±	+	+	+	+	0	+	0	+	0	+	0	0	+	+	0	+	9.6	37
L-356	*L. casei*	V300	±	+	+	+	+	+	+	0	+	0	+	0	0	+	+	0	+	8.9	37–45
L-326	*L. casei*	(LD-5) 9595	0	+	+	+	+	0	+	0	+	0	+	0	0	+	+	0	+	9.7	37+
L-354	*L. casei*	Ld-5	0	+	+	+	+	±	+	0	+	0	+	0	0	+	+	0	+	8.8	37±
L-379	*L. casei*	Ldg	±	+	+	+	+	+	+	0	+	0	+	0	0	+	+	0	+	8.6	37±
L-330	*L. casei*	1438	0	+	+	+	+	0	+	0	+	0	+	0	0	+	+	0	+	8.7	37
L-339	*L. casei*	ZBL	0	+	+	+	+	+	+	0	+	0	+	0	0	+	+	0	+	8.6	37–45
L-340	*L. casei*	6557	0	+	+	+	+	+	+	0	+	0	+	0	0	+	+	0	+	8.7	37
L-369	*L. casei*	V349	±	+	+	+	+	+	+	0	+	0	+	0	0	+	+	+	+	8.9	37–45
L-377	*L. casei*	7649	±	+	+	+	+	+	+	0	+	0	+	0	0	+	+	0	+	8.8	37±
L-378	*L. casei*	4796	±	+	+	+	+	+	+	0	+	0	+	0	0	+	+	0	+	8.7	37±
L-311	*L. helveticus*	L43	±	+	+	+	+	+	+	0	+	0	+	0	0	+	+	+	+	9.6	37−
L-391	*L. helveticus*	B-442	±	+	+	+	+	+	+	0	+	0	+	0	0	+	+	0	+	9.4	37
L-313	*L. delbrueckii*	5R9573	±	+	+	+	+	+	+	0	+	0	+	0	0	+	+	0	+	9.6	37±
L-336	*L. delbrueckii*	1358	±	+	+	+	+	+	+	0	+	0	+	0	0	+	+	0	+	9.6	37
L-380	*L. delbrueckii*	Ld4913	±	+	+	+	+	+	+	0	+	0	+	0	0	+	+	0	+	8.5	37
L-381	*L. delbrueckii*	7R5676	±	+	+	+	+	+	+	0	+	0	+	0	0	+	+	0	+	8.5	37
L-392	*L. delbrueckii*	B-445	±	+	+	+	+	+	+	0	+	+	+	0	0	+	+	0	+	9.2	37
L-309	*L. bulgaricus*	7995	0	+	+	+	+	0	+	0	+	0	±	0	0	+	+	0	+	9.2	37−
L-310	*L. bulgaricus*	7995	±	+	+	+	+	0	+	0	+	0	+	0	0	+	+	0	+	9.6	37−
L-373	*L. acidophilus*	(milk strain)	0	+	+	+	+	+	+	0	+	0	+	0	0	+	+	+	+	9.3	37
L-641	(oral strain)		±	+	+	+	+	±	+	0	+	0	+	0	0	+	+	+	+	9.2	37+
L-652	(oral strain)		0	+	+	+	+	+	+	0	+	0	+	0	0	+	+	+	+	9.0	37±
46A	(oral strain)		0	+	+	+	+	+	+	0	+	0	+	0	0	+	+	+	+	8.8	37±

All strains listed produced acid, coagulation, and reduction in litmus milk and grew abundantly in TJY broth.
* Acid production, usually within a few days, is indicated by a plus (+) sign; (±) indicates a slight change in the color of the indicator by the end of 14 days. No color change at anytime is shown by a zero (0).

TABLE 4.—*The dissimilarity of fermentative characteristics of representative* Lactobacillus *strains not exhibiting an* F *antigen.*

Laboratory Number	Species or strain designation	Strain number	Arabinose	Rhamnose	Sorbose	Glucose	Lactose	Sucrose	Trehalose	Melibiose	Cellobiose	Raffinose	Melezitose	Inulin	Dextrin	Mannitol	Sorbitol	Dulcitol	Salicin	Titrable acidity in ml of *N*/10 NaOH
L-325	*L. casei*	334	0	0	0	+	+	+	+	0	+	0	+	0	0	+	0	0	+	9.4
L-388	*L. bulgaricus*	2889	0	0	0	+	+	0	±	0	0	0	0	0	0	0	0	0	0	0.6*
L-306	*L. delbrueckii*	LD-3	0	0	0	+	0	+	+	0	+	0	+	+	+	+	+	0	+	8.0
L-329	*L. acidophilus* (Döderlein's bacillus)	9857	0	0	0	0	0	±	0	0	0	0	0	0	±	0	0	0	0	0.4*
L-612	Vaginal strain	Lb-6	±	0	0	+	+	+	+	0	+	0	+	0	0	+	+	0	+	9.1
L-615	Fecal strain		±	0	+	+	+	+	+	0	+	0	+	+	0	+	+	+	+	8.8
L-338	*L. odontolyticus*	450.	±	+	+	+	+	0	+	0	+	0	+	0	0	+	+	0	+	9.5
L-345	*L. acidophilus* (oral strain)	L5	0	0	0	+	0	0	0	0	0	0	+	0	0	+	0	0	0	4.3
L-347	*L. acidophilus* (oral strain)	S42B	0	+	+	+	+	+	+	0	+	0	+	0	0	+	+	+	+	8.9

In addition to the carbohydrates listed all strains except L-306 grew and produced acid, coagulation, and reduction in litmus milk, and all but L-388, L-306, and L-329 grew abundantly in TJY broth.
* Growth in this medium by L-388 and L-329 was scanty; this may account for the low acid productivity.

tainty that the 9 major antigens in lactobacilli studied to date are distinct and separate entities.

Correlation of antigenic with fermentative characteristics.—Since the display of antigenicity by a bacterium is only one of many attributes, the correlation of this trait with other characteristics seemed quite pertinent. Table 3 indicates the similarity of fermentative and other characteristics of lactobacilli exhibiting an *F* antigen. Listed in this table are only the 17 carbohydrates and derivatives which seemed to be of some differential value. Reaction in milk, titrable acidity, reaction on blood agar, and the optimal temperature for growth were other determinations thought to be differential as indicated in tables 3, 4, and 5. All *L. casei* strains with the A.T.C.C. number 7469, as illustrated in table 3, including the three strains designated as BC-1, ϵ, and V300, which presumably all came from the same parent culture, exhibited an *F* antigen. All these 9 cultures showed only slight variation in their fermentation reactions, titrable acidity, and action on blood agar (which was observed to be a faint or bleached zone circumventing the individual colony in almost all instances, but as a whole this reaction was somewhat variable). All *L. casei* cultures with an *F* antigen as well as other lactobacilli exhibiting an *F* antigen also reacted very similarly on the carbohydrates tested. Only the fermentation of sucrose showed some variation. Likewise, three cultures listed as oral strains as well as an *L. casei* (L-369) which originally was isolated also from the oral cavity all manifested an *F* anti-

TABLE 5.—*The fermentative characteristics of representative strains having different demonstrable major antigens.*

Laboratory number	Species or strain designation	Strain number	Antigen designation	Arabinose	Rhamnose	Sorbose	Glucose	Lactose	Sucrose	Trehalose	Melibiose	Cellobiose	Raffinose	Melezitose	Inulin	Dextrin	Mannitol	Sorbitol	Dulcitol	Salicin	Titrable acidity in ml of *N*/10 NaOH
3A1SB	Oral strain		*AB*	0	+	+	+	+	+	+	0	+	0	+	0	0	+	+	0	+	8.9
4A2SD	Oral strain		*C*	0	0	+	+	+	+	+	0	+	0	+	+	0	+	+	0	+	8.7
45A	Oral strain		*BD*	0	0	+	+	+	+	+	0	+	0	0	+	±	+	+	0	+	8.7
EH22G	Oral strain		*E*	±	+	0	+	+	+	+	0	+	+	+	0	±	+	+	0	+	8.6
L-320	*L. casei*	7469	*F*	0	+	+	+	+	0	+	0	+	0	+	0	0	+	+	0	+	9.7
L-304	*L. plantarum*	10012	*G*	+	0	0	+	+	+	+	+	+	0	+	0	+	+	+	0	+	9.1
L-323	*L. casei*	393	*H*	0	0	0	+	+	0	+	0	+	0	+	0	0	+	±	0	+	9.6
L-641	Oral strain		*FI*	±	+	+	+	+	±	+	0	+	0	+	0	0	+	+	+	+	9.2

All strains grew and produced acid, coagulation, and reduction in litmus milk and grew abundantly in TJY broth.

gen and showed a similar fermentation pattern with the additional capability of fermenting dulcitol. Almost all other strains with an *F* antigen failed to ferment this hexose alcohol on repeated testing. As a generalization, the ability on the part of lactobacilli to produce acid from both rhamnose and sorbose in sufficient quantities to change the bromcresol purple indicator seemed to be associated with the presence of an *F* antigen in the 30 strains studied. Within a rather narrow range, the celullar and colonial morphology of all strains listed in table 3 were quite similar. The cells were found to be gram-positive, palisading rods usually measuring 1.0 to 2.0×0.3 to $0.5\ \mu$, while the colonies were convex, smooth, and glistening.

By contrast to the consistent similarity among the strains just discussed, the cultures listed in table 4 did not have an *F* antigen and reacted much differently for the most part in their polysaccharide-fermenting ability. The strains considered in this table are merely representative of a great number of lactobacilli which showed a similar lack of correlatable traits. For comparison of other attributes of lactobacilli manifesting the different major antigens studied to date, representative strains (including 4 used by N. B. Williams) are listed in table 5. It is notable that none have identical characteristics, although strains having *AB*, *F*, or *FI* antigens do not differ markedly from one another in their other characteristics.

Antigen stability studies.—In order to learn more of the intrinsic nature of certain major antigens exhibited by lactobacilli, cell suspensions as well as growing cultures were subjected to various altered environmental conditions to determine whether this might influence their antigenicity.

Washed lactobacillus cells were subjected to both mild and severe physical and chemical treatment. Water suspensions of two strains (L-301 and L-326, both having an *F* antigen) were maintained at 65 C for two hours, other suspensions of these strains were kept in boiling water for two hours, while still others were subjected to steam in the autoclave at 15 pounds' pressure for 15 minutes. Despite such physical treatment, these cells retained their antigenicity as shown by antibody production in rabbits when used as vaccines and by cell agglutination to full titer in specific antiserum. Subjecting other cell suspensions (L-320 and L-340) to the other extreme in temperature, freezing for several days at −7 C, likewise did not alter the ability of the cells to agglutinate in homologous serum. Suspending (L-301 and L-326) cells in 50% and later in absolute ethyl alcohol for 24 hours at 37 C also did not affect their antigenic nature. Extraction of polysaccharide from the cells of these same strains by boiling in *N*/16 hydrochloric acid for 10 minutes likewise did not influence the *F* antigen. However, placing the cells in *N*/1 hydrochloric acid for 24 hours at 37 C completely destroyed the antigen as determined by agglutination tests. Ten minutes' boiling of the cells in *N*/10 sodium hydroxide apparently lysed about 60% of them, but those remaining still manifested their usual antigen as before, although there was a slight tendency toward autoagglutination.

Cells grown under various environmental conditions were also observed for changes in antigenicity. Cultures frozen in solid carbon dioxide at a temperature of approximately −78 C for 5 months as well as cultures rapidly transferred through TJY broth on alternate days over an 8-week period showed no alteration of their antigenic characteristics. Cultures grown in a variety of

mediums such as the partly synthetic medium used for acid titration and veal infusion broth containing 0.5% of various sugars all produced cells manifesting the same major antigens as those grown in the commonly used TJY broth. Strains (L-301 and L-641) were also grown in TJY broth to which had been added sublethal amounts of sodium chloride, sodium fluoride, or phenol.* Cells grown in the presence of such inhibiting agents after 16 serial transfers over a 6-week period showed no visible degree of granularity or roughness, and agglutination tests indicated these strains had retained their original antigenic components of *F* and *FI*, respectively.

The growth of lactobacilli (strain L-301) in TJY broth to which anti-*F* serum had been added to make a 10% serum broth resulted in a very granular growth at first. Autoagglutination of these cells even when repeatedly washed rendered agglutination tests unreadable. On repeated transfers through such antiserum broth, the growth became less granular and cells later developed which no longer autoagglutinated, making it feasible to perform agglutination tests. Such tests revealed a complete absence of the *F* antigen. Subsequent growth of such altered strains in TJY broth without the antiserum resulted in the continued production of cells which likewise did not manifest an *F* antigen. In the case of one strain, however, the cells eventually regained their former antigenic traits. Another strain has not done so to date, despite some 46 transfers over a period of 17 months. More data regarding this rather unique phenomenon is to be presented in a later publication.

Certain cultures of lactobacilli were serially transferred through TJY broth to which increasing amounts of potassium penicillin had been added. As surmised, this caused the strains to develop various degrees of resistance toward this antibiotic. Induction of such resistance resulted in an *L. casei* strain (L-301) which was just able to grow in the presence of 10 μg of crystalline penicillin per ml of TJY broth, as compared to a natural resistance of a little under 0.5 μg per ml of the parent culture.† An *L. plantarum* strain (L-304) became resistant to 20 μg per ml of medium as compared to 1μg per ml for the parent strain. By contrast, the two oral strains L-641 and L-652, though transferred with the same frequency as the others, had developed a tolerance for only 1 μg and slightly less than 1 μg, respectively, per ml of TJY broth. This was only twice their original natural resistance of 0.5 μg per ml. All 4 of these strains after the various degrees of penicillin tolerance had been induced were found to continue manifesting their major antigens as well as fermentation reactions in the same manner as their parent strains.

Attempt at bacteriophage isolation.—The high degree of lytic specificity of bacteriophage for certain bacterial strains prompted the search for phage capable of lysing lactobacilli. It was conjectured this lytic specificity might have some correlative bearing on the somatic antigens found in lactobacilli analogous to the observations regarding phage specificity and *Salmonella* antigenicity as reviewed by Wilson and

* A sublethal quantity of NaCl in TJY broth was a 7.5% solution, whereas 25 parts per million of the fluoride ion in the broth proved to be sublethal for the lactobacilli tested, and a 0.17% solution of phenol in TJY broth was found to be sublethal at first, but could be increased slightly later.

† According to Cutter Laboratories of Berkeley, California, preparing this antibiotic, one Oxford unit of potassium penicillin G was equivalent to 0.6275 μg of the crystalline substance.

Miles (1946). Testing of substances in which lactobacilli are commonly found such as saliva, feces, and sauerkraut failed to reveal bacteriophage lytic for the several strains of lactobacilli used in the tests. It was of interest to note that Weiss and Rettger (1934) were likewise unable to find bacteriophage specific for their *L. bifidus* strains. However, Kopeloff (1934) reported using a phage active on certain strains of both *L. acidophilus* and *L. bulgaricus*. Furthermore, a search through earlier reports revealed that Sierakowski and Zajel (1924) obtained bacteriophage active against *B. acidophilus odontolyticus*, the bacteria having been isolated from saliva and carious teeth.

DISCUSSION

Correlation of antigenic traits.— Among the many strains of lactobacilli accumulated and studied in the laboratory collection, few proved to be more interesting than the *L. casei* strain A.T.C.C. 7469. The original culture, according to McCoy (1949), came to the University of Wisconsin in 1926 from Ernest Pribram of the Kral Museum in Vienna. It was designated as *Bacillus casei* because of its apparent similarity to the *B. casei* of Freudenreich reported in 1904. However, it was called *B. casei* ε in early publications instead of the correct designation, Freudenreich α. This was unfortunate, because the incorrect designation has persisted and according to the 4th, 5th, and 6th editions of Bergey's Manual, the Freudenreich ε strain is classified as *L. helveticus*, whereas the α strain is *L. casei*. Sherman verified that the culture from Vienna was *L. casei* and not *L. helveticus;* later Fred designated it as BC-1. In 1939, Strong sent this *L. casei* strain to the American Type Culture Collection where the number 7469 was affixed. With this designation, the strain attained considerable prominence in microbiological assays of vitamins and amino acids. Unfortunately however, most published reports of such assays in which this or some other *L. casei* strain was used have failed to indicate the exact strain employed. The assumption probably has been that all strains labeled *L. casei* are identical, but this is a presupposition very often found to be untenable. As will be emphasized later, many strains designated as *L. casei* show considerable differences in their antigenic as well as in other characteristics. Hence, it has become a problematical point as to whether their nutritional requirements would differ correspondingly.

Strains of *L. casei* designated 7469, BC-1, or ε, collected from various laboratories including the A.T.C.C. and the University of Wisconsin laboratories, were found to be alike in almost all respects. It was particularly gratifying to observe that the major antigen *F* was manifested by all 9 such strains. Furthermore, all reacted identically in their carbohydrate-fermenting abilities with the exception of three strains found to react on an additional sugar. This difference may not be very significant, since after years of cultivation on a variety of substrates, minor variation such as this increased capability of sucrose fermentation in these few strains may well have occurred. Many similar instances of strain variation in fermentation could be cited from the literature. The important observation, however, that a more fundamental character such as a major antigen has remained unaltered is of considerable interest and in all probability has valuable implications. That 9 other strains of *L. casei* (not known to be progeny of the previously discussed strain) all showed the presence of the same major antigen with a similarity of fermenta-

tion pattern, seemed significant mainly by contrast to the 8 additional *L. casei* designated-strains found to exhibit no such antigen and also to differ in their fermentative abilities. Other lactobacilli possessing an *F* antigen and having a correspondingly similar fermentation pattern were found to be designated as *L. helveticus*, *L. bulgaricus*, *L. delbrueckii*, and *L. acidophilus*. A few strains with similar traits were discovered among oral lactobacilli. Yet, it is well to promptly point out that though both *L. helveticus* strains tested showed an *F* antigen, only 2 of 5 *L. bulgaricus* strains, 5 of 8 *L. delbrueckii* strains, 1 of 17 *L. acidophilus* strains, and 4 of 93 oral lactobacilli exhibited this common *F* antigen. The strains which did not possess this particular antigen also differed in other traits, thus further emphasizing their basic dissimilarity.

In relation to the problem of dental caries causation, it was of considerable interest to observe the presence of an *F* antigen in at least a few lactobacilli found in the oral cavity of individuals with carious teeth. One of these strains (L-641 with the antigenic formula *FI*) was isolated relatively recently (October, 1947), whereas the others had been cultivated on laboratory medium for a decade or more. One of the latter was received indirectly from Kulp as a "caries culture." It had been studied by Rosebury as #59, by Curran in 1931 as #95, and by Tittsler and Rogosa as the V349 *L. casei* strain. It was notable that all of these oral lactobacilli had the capability of fermenting the hexose alcohol, dulcitol, unlike almost all other oral strains tested and unlike other strains having an *F* antigen (with the exception of strains L-311 and L-373). The full significance of this unique fermentative trait as well as the presence of lactobacilli with an *F* antigen in the oral cavity is not apparent at this time.

In the attempted correlation of antigenic characteristics of these bacteria, only the ability to ferment rhamnose and sorbose seemed associated with the presence of an *F* antigen. Every strain exhibiting this major antigen was observed to ferment both of these pentoses. Nevertheless, there were found 4 other stock strains capable of fermenting both these sugars which exhibited antigenic components *A* or *AB* instead of *F*. A considerable number of other antigenically unrelated strains fermented either one or the other but not both of these same two simple carbohydrates. This capability of fermenting both rhamnose and sorbose by all strains with an *F* antigen and by some with an *A* or *AB* antigen may have some special significance though not entirely clear at present.

Observation of fermentation with mere acid production over a certain threshold level, as shown by a change in the indicator color, can be improved upon quantitatively by measuring the total titrable acid produced. Camien et al (1947) showed that a culture like *L. casei* 7469 produced less acid from rhamnose, mannitol, and sorbitol, respectively, than from glucose. Theoretically, glucose can yield two moles of lactic acid per mole when acted upon by a homofermentative microorganism, and in actual titration the amount of acid produced from glucose by strains having an *F* antigen approximated this quantity, though no attempt was made to identify the acid. Such relatively large amounts of acid produced by these strains were in distinct contrast to a group of other strains found capable of producing only half as much titrable acid. These latter lactobacilli were similar, at least in this respect, to the "weakly acidogenic" strains (as L-345) of Sullivan et al (1939). Moreover,

these weakly acidogenic strains seldom seemed to exhibit a high degree of antigenicity as determined by cell agglutination in both homologous and heterologous antiserums.

Because of the nature of distribution of this one major antigen *F* among lactobacilli isolated from diverse habitats and designated as different species, some explanatory suggestions are probably in order, though none can be completely substantiated by evidence. The simplest explanation is that possibly all strains manifesting an *F* antigen are really of the same species, perhaps *L. casei*, especially since 10 different well known strains of this species exhibited the same antigen. It would then be necessary to assume that all strains not labeled *L. casei* but still possessing this same antigen had been misclassified at the time of isolation or some time thereafter. This idea may be explanatory in some instances but it may seem somewhat untenable for all strains concerned. Inasmuch as there are the 8 other strains all named *L. casei* which did not manifest an *F* antigen and also differed in their fermentation reactions, it would be necessary to presuppose these also had been misclassified or differed for some other reason. It may be possible, but seems unlikely, that such a large proportion of well known *Lactobacillus* strains should have been misnamed as to species. A second explanation might be that certain strains of lactobacilli, after being cultivated in artificial medium over a period of years, may have evolved by selection pressure to reach a common, stable form which in this particular instance manifests an *F* antigen. In support of this contention it might be mentioned that lactobacilli on initial isolation, particularly from the oral cavity of man or animals, are often found to be in a rough phase but later seem to dissociate to a smooth and more stable form. This concept is somewhat of a generalization of the view expressed by Pederson (1947) that *L. acidophilus* on cultivation becomes indistinguishable from *L. casei*. Relative to this dissociation phenomenon, it should be stated that all cultures with the major antigen *F*, in all probability had been carried in laboratory medium for at least a decade. The one exception, L-641, was isolated a few months before being tested for an *F* antigen though it was almost a year later that it was found also to possess an *I* antigen. It should be interesting to note whether the *I* antigen will persist after this strain has been cultivated on laboratory medium for a decade or so. On the other hand, a great number of other stock cultures had been maintained on similar artificial medium for more than a decade and they exhibited neither an *F* antigen nor any other major antigen studied thus far. A third explanatory comment, not necessarily in conflict with either of the first two, might be put in the form of a question: Is it not conceivable that a group of lactobacilli, regardless of their immediate ecological habitat and despite a few minor variations in other characteristics, may possess in common some cellular chemical constituent which serves as a haptenic substance capable of imparting an identical character to each of the strains concerned? On the basis of such a concept, which requires more sustaining evidence, a knowledge of the antigenic pattern of lactobacilli by simple antigenic analysis would seem to be of considerable value in differentiation, identification, and classification of bacteria belonging to the *Lactobacillus* group.

Stability of antigenic traits.—The presence of a common antigen among a considerable number of ostensibly different lactobacilli made it pertinent to learn just how stable this particular

antigen was and under what environmental circumstances it might be suppressed or lost and regained.

The *F* antigen must be considered as heat stable when cells can be boiled or steamed under pressure and still exhibit the same antigen to full titer in agglutination tests as well as in the ability to stimulate specific antibody production in rabbits. Upton and Kopeloff (1932) tested their lactobacillus type antigen by boiling in water for an hour. Such treatment, they concluded, did not alter the cells' agglutinability. This sole report in the literature on such antigen testing supports the contention that these somatic antigens in lactobacilli contain no heat labile fraction.

The major antigen studied was also found to be stable to the dehydrating and lipoid solvent effect of absolute alcohol. Neither the removal of polysaccharide by boiling in *N*/16 hydrochloric acid for 10 minutes nor the boiling in *N*/10 sodium hydroxide for a like period altered the antigenic expression as revealed by agglutination tests. In the future, other tests including enzymatic digestion of the bacterial cells should be tried in the attempt to learn more of the intrinsic traits of these antigens in lactobacilli.

Repeated cultivation of strains with an *F* antigen in broth containing sublethal quantities of phenol, sodium fluoride, or sodium chloride resulted in cells which still manifested this antigen to the same degree as before. Causing 4 different strains of lactobacilli to become penicillin-resistant revealed no modification of their antigenic pattern. Thus, even with such an induced condition as penicillin resistance, which Demerec (1948) considers to be a mutation and selection phenomenon, the character of *Lactobacillus* antigenicity has remained stable and unchanged. It had been thought that if these lactobacilli could have been induced to become highly resistant to the antibiotic, some of their other traits including antigenicity might have been altered analogous to the morphological and tinctorial changes which occurred in a *Staphylococcus* strain which had its resistance to penicillin increased 60,000 times as reported by Bellamy and Klimek (1948).

Strains cultivated in the presence of specific antiserum which developed progeny no longer manifesting the *F* antigen remained unaltered in all of their other characteristics. The apparent suppression or loss, at least temporarily, of a major somatic antigen among lactobacilli constitutes a rather interesting immunological phenomenon, though the mechanism involved is highly uncertain at this time.

SUMMARY

An attempt at clarifying the subgeneric relationship of lactobacilli was made by determining the antigenic pattern and correlating it with other differentiating characteristics of these bacteria. Some 250 strains of lactobacilli, many with species designations, were studied by means of cell agglutination and agglutinin absorption tests. The presence of major antigens as evidenced by agglutinating titers over 10^{-3} was observed in a number of well known species of the *Lactobacillus* group. They were designated by alphabetical letters, continuing the method of designation started by Williams in his study of oral lactobacilli in this laboratory.

The major antigen, designated *F*, was observed in a number of strains isolated from a variety of different sources including the oral cavity. Many of these strains had been given various species names by other laboratories. It was suggested that possibly all strains with an *F* antigen, especially since their other characteristics were very similar,

were really the same species, perhaps *L. casei*, and those labeled as other species were simply misclassified. On the other hand, by long cultivation certain strains might have evolved to reach a common, stable state which happened to manifest this particular *F* antigen.

Additional major antigens designated *G*, *H*, and *I*, were observed in certain other lactobacilli.

To gain information about the more intrinsic nature of one of these major antigens, cells with an *F* antigen were subjected to boiling, steaming under pressure, freezing, absolute alcohol, *N*/16 hydrochloric acid, and *N*/10 sodium hydroxide. In spite of these treatments, they still maintained their antigenicity. Growing cells transferred through broth having sublethal amounts of phenol, sodium fluoride, sodium chloride, or penicillin likewise did not show a loss of their *F* antigen. After serial transfers in broth containing a specific antiserum a few strains did not manifest their *F* antigen, although retaining their other traits. By repeated transfers through broth without the antiserum, one strain reverted to its former antigenic state, but another has not done so to date.

Major antigens found in lactobacilli were observed to be rather stable, separate entities, and antigenic analysis has been demonstrated to be of considerable value in the differentiation and classification of certain bacteria belonging to the *Lactobacillus* group.

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RICKETTSIAE IN THE BLOOD OF MICE AND RATS INFECTED EXPERIMENTALLY WITH TYPHUS

W. NYKA

From the Department of Medical Research, General Hospital, Mexico, D. F.

Typhus and brucellosis, though etiologically and clinically entirely different, show, nevertheless, a striking similarity in some of the features of their pathology. In both, for instance, the spleen contains the causative agent of the respective disease and the blood is virulent in a high percentage of cases. In a study to be published[1] it is recorded that in rats infected with heavy doses of *Brucella abortus*, there is a close relationship between the organisms found in the spleen and those encountered in the blood of the animals. This paper brings evidence that a similar though less apparent relationship exists between the presence of rickettsiae in the spleen and the virulence of the blood of animals infected with typhus.

MATERIALS AND METHODS

Forty-seven mice and 92 rats were infected intranasally[2] with rickettsiae of murine type and 24 mice with rickettsiae of classic type. An additional series of 6 rats were treated with X rays[3] and two days later infected intraperitoneally with rickettsiae of murine type. The mice were anesthetized with ether and exsanguinated shortly before death was expected to occur. The intranasally infected rats were sacrificed in the same way, 48 animals 60 hours and 44 animals 72 hours after infection. The intraperitoneally infected rats were exsanguinated between 72 and 84 hours after infection. The brain, kidneys, liver and spleen of these animals were taken for histological examination, the sections being stained with methyl violet and metanil yellow.[4] Giemsa-stained blood smears were made of all animals, either directly or after short centrifugation at low speed. Other mice were inoculated intranasally with the blood of rats infected with rickettsiae of murine type and with that of mice infected with rickettsiae of either type, and the lungs of mice of these series infected with rickettsiae of murine type which died were used for intranasal inoculation of 12 rats. These rats died or were killed 72 hours after infection. An antigen was prepared from their lungs, and the agglutination and complement fixation tests were carried out according to the method in use in this Department (Dr. R. Silva Goytia).

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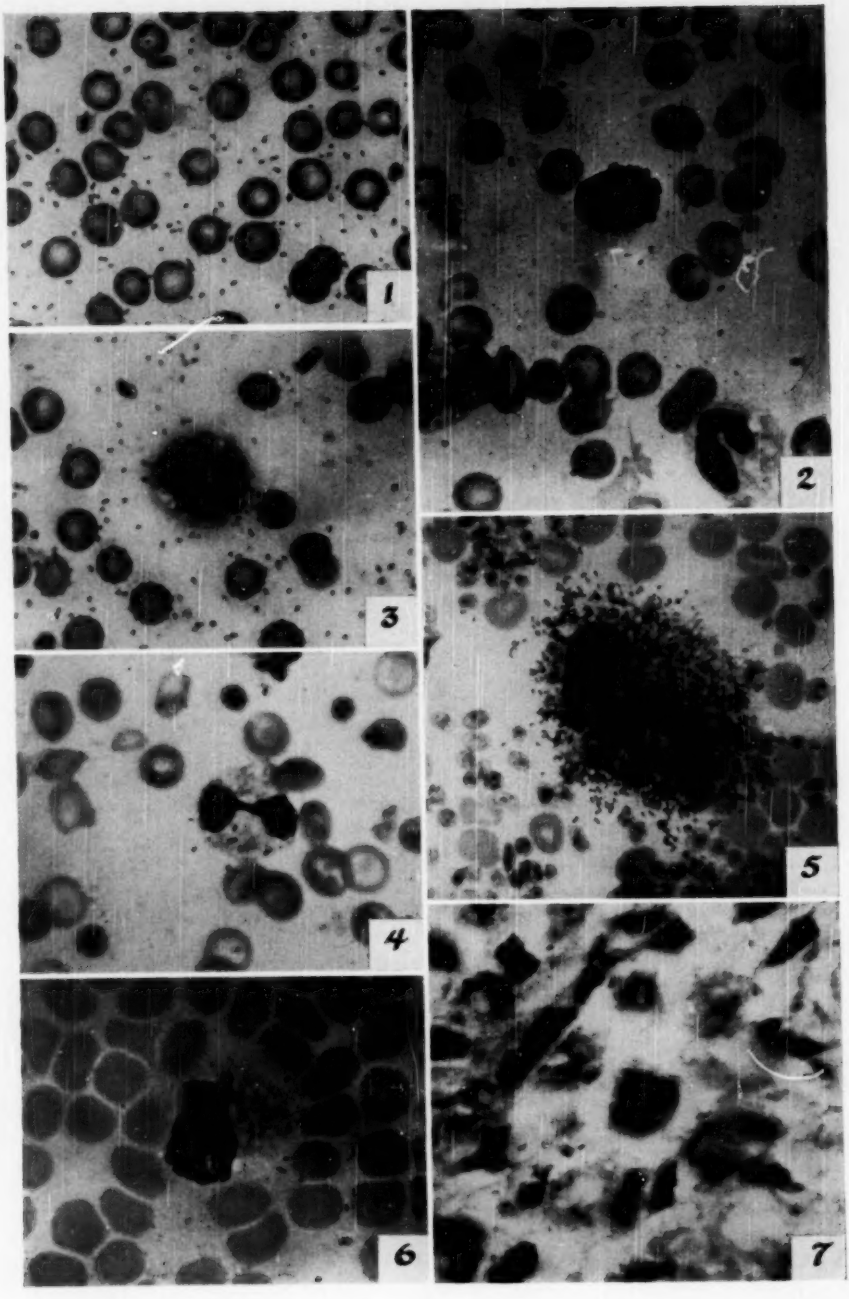
RESULTS

Rickettsiae were found in the blood of 31 out of 47 mice (65.9%) and of 19 out of 92 rats (20.6%) infected intranasally with rickettsiae of murine type. They were present in the blood of all 24 mice infected in the same way with rickettsiae of classic type and in that of all 6 rats infected intraperitoneally with rickettsiae of murine type. The number of rickettsiae encountered in individual animals was very variable and was few in most of the animals. The organisms were found with relative ease in a few animals; and in one rat infected intranasally with rickettsiae of murine type and in two mice infected by the same route with rickettsiae of classic type, they were as numerous as is shown in figure 1. Generally, beside being more frequent, rickettsiae of classic type were also more numerous in the blood of mice than organisms of murine type. Similarly, rickettsiae of the latter type were less frequent in the blood of intranasally infected rats than in that of irradiated and intraperitoneally infected animals, just as they were less commonly found in intranasally infected rats than in mice infected by the same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route. Great differences were also noted between the various series of animals examined. In some series of 4 or 6 mice infected with rickettsiae of murine type, all animals were positive. In others only 1 to 3 of them had rickettsiae in their blood, and in others all animals were negative. In the series of rats, never more than 4 out of 8 animals had rickettsiae in their blood.

The organisms were either free (fig. 1) or inside polymorphonuclear leucocytes (fig. 3), monocytes and reticular cells (fig. 5). Polymorphonuclear leucocytes and reticular cells were the most frequently and the most severely infected. Monocytes containing rickettsiae were a less common finding. Phagocytes usually contained a moderate number of loosely arranged, single rickettsiae (fig. 3), while many of the reticular cells were filled to capacity with them (fig. 5). Both extra- and intracellular rickettsiae were normal in appearance, and so were the polymorphonuclear leucocytes and monocytes harboring them. On the contrary, many of the infected reticular cells showed signs of degeneration; their cytoplasm was abundantly vacuolated and sometimes fragmented, and their nuclei were either pycnotic or contained clear areas, as if void of chromatin. Not infrequently such cells were broken up and formed small agglomerations of cellular debris among which numerous rickettsiae were found.

A noteworthy feature in the blood of the two mice infected with classic rickettsiae in which the organisms were particularly numerous was the strikingly great number of paired forms, which were more numerous than cocci, coccobacilli and rods put together.

A full histological description of the spleens of the irradiated and intraperitoneally infected rats will be given in another study. Great numbers of rickettsiae were found in all 6 of them. The organisms were either free or inside polymorphonuclear leucocytes, monocytes, adventitial cells of blood capillaries and littoral cells of venous sinuses, the latter still in their place or desquamated and floating free in the sinuses (fig. 7).

The results of the intranasal inoculation of normal mice with the blood of infected mice and rats depended on the number of rickettsiae it contained. If the organisms were numerous, the animals developed lesions in all respects similar to those observed in mice infected with a suspension of lungs, the only difference being that the resulting bronchopneumonia was brought about more slowly and that only a certain number of the animals died, usually between 6 and 9 days after infection. At the autopsy, the lungs of animals which

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EXPLANATION OF FIGURES

FIG. 1.—Murine rickettsiae in the blood of a rat. ×1000.

FIG. 2.—Free rickettsiae of various types in the blood of a typhus patient examined 8 days after the onset of the disease. ×1000.

FIG. 3.—Polymorphonuclear leucocyte containing a fairly large number of rickettsiae of various types and free organisms scattered among the red blood cells. Taken from the same patient as figure 2. ×1000.

FIG. 4.—Polymorphonuclear leucocyte with a few rickettsiae of various types and two free coccobacillary forms. Taken from the same patient as figure 2., taken from the blood of a rat. ×1000.

FIG. 5.—Reticular cell filled with rickettsiae of murine type. ×1000.

FIG. 6.—Reticular cell with a few rickettsiae in the cytoplasm and 2 to 3 extracellular organisms, taken from the same patient as figures 2 and 4. ×1000.

FIG. 7.—Desquamated reticular cells in a venous sinus of the spleen of a rat, one of which is filled with rickettsiae of murine type. ×1000.

died presented the typical appearance of rickettsial bronchopneumonia. In animals killed at various intervals after infection, foci of consolidation were found all over the lung but mainly around the hilum. Control animals, inoculated with the blood from infected animals found negative or with that of normal animals, regularly showed no alterations in their lungs.

The agglutination and complement fixation tests carried out with the antigen prepared from the lungs of rats infected with the strain of murine rickettsiae recovered from the blood of mice were found strongly positive.

The detection of rickettsiae in the blood of experimentally infected animals suggests that a thorough search might eventually lead to similar findings in human blood. As a matter of fact, in the blood of a series of typhus patients organisms have been found which, by their size, morphology and position in the blood, were very suggestive of rickettsiae. Like rickettsiae they appeared as cocci, coccobacilli, diplococci and fine rods and were either free (fig. 2) or inside polymorphonuclear leucocytes (fig. 4), monocytes and reticular cells (fig. 6). Their number was very small in most of the cases, and a careful, patient search and perfectly stained, clean preparations were necessary to detect them.

DISCUSSION

The experiments recorded show that rickettsiae of either type can be found in the blood of mice and rats, no matter whether infected intranasally or intraperitoneally. It appears, therefore, that any debilitating treatment as used by Zinsser and Castañeda, who have seen rickettsiae in the blood of rats which had received subcutaneous injections of benzol previous to the inoculation,[5] is not necessary to secure the passage of rickettsiae into the blood. It must be added, however, that the incidence of rickettsiae seems to be increased by such a treatment, as appears from the fact that the blood of all 6 irradiated and intraperitoneally infected rats contained rickettsiae as contrasted to finding the organisms in only a relatively small percentage in intranasally infected animals, whose resistance had not been lowered by any previous treatment. This observation would be of some biological significance if the difference in the findings in the two series of rats was due exclusively to the irradiation and not, as is possible, to the different route of infection of the animals.

Another factor which influences greatly the appearance of rickettsiae in the blood of rats is the time which elapses between the infection and the bleeding of the animals, as is clearly demonstrated by the fact that all 48 rats examined 60 hours after intranasal infection were found negative, while 19 out of the 44 animals (43.2%) bled 72 hours after infection had rickettsiae in their blood. It appears, therefore, that the spread of the organisms takes place during the very last hours of life of the animals. This observation finds an explanation by the way in which rickettsiae develop in the lungs of rats. It has been shown[6] that the most active development of the organisms is observed shortly before death occurs and that it culminates in the appearance of great numbers of extracellular rickettsiae which invade the cellular interstices and lymph spaces, from which, in all probability, the organisms find their way to the circulating blood.

The striking similarity of the organisms found in the blood of a series of typhus patients (figs. 2, 4 and 6) with

5. Zinsser, H. and Castañeda, M. R. 1930, J. Exper. Med. **52**: 649–659.
6. Nyka, W. 1947. Am. J. Path. **23**: 843–857.

those encountered in the blood of animals and identified beyond any possible doubt as rickettsiae of typhus justifies their presentation in this study of rickettsiae in the blood. Their rickettsial nature has not been established by serological tests, mainly because the two cases of typhus suitable for such investigation were examined at the beginning of these studies, when the new method of isolation of rickettsiae by means of the mouse lung was not yet used. Since the number of rickettsiae encountered in the blood of these two cases was not lower than that observed in the samples of blood of mice and rats, the inoculation of which produced the most typical typhus bronchopneumonia, it is hoped that similar results might be obtained with the blood of suitable typhus patients and the help of the new method of isolation of rickettsiae from the blood. In this way, the mystery which still surrounds the problem of the virulence of the blood of typhus patients would be cleared up and evidence provided that the early observations of Ricketts and Wilder[7] were correct.

For our knowledge of the pathogenesis of typhus it is important to determine exactly the source of infection of the blood and to clear up the mechansism by which rickettsiae gain access to the circulation. There seems to be no doubt that in experimentally infected animals the site of infection, lung or peritoneum, flooded with masses of rickettsiae, represents an important source of spread of the organisms. In intranasally infected rats rickettsiae seem to find their way to the circulating blood directly from the lung, but in intraperitoneally infected animals the organisms may reach the circulation either directly, through the lymph spaces of the peritoneum, or indirectly, by the intermediary of the spleen, as appears from the close study of the infected reticular cells found in their blood. Broadly speaking these cells may be: (1) endothelial cells of capillaries and small blood vessels, (2) mesothelial cells of the peritoneum, (3) alveolar cells of the lung and (4) reticuloendothelial cells. A close examination of these cells (fig. 5) shows that their morphology is different from that fairly characteristic of endothelial cells of the vascular system. Besides this, endothelial cells of capillaries filled with rickettsiae are not usually found in the lungs of intranasally infected mice and rats,[4,6] while cells of the type of the one represented in figure 5 are far from being unusual in their blood. Desquamated mesothelial cells packed with rickettsiae are frequent in the exudate of irradiated and intraperitoneally infected rats. But these cells are eventually destroyed as appears from the presence of degenerated cells and abundant cellular debris in the exudate of the peritoneal cavity. The opinion that the reticular cells are in reality alveolar cells of the lung can easily be refuted by the argument that similar cells have been found in the blood of animals infected intraperitoneally, the lungs of which were found to be normal. Furthermore, it has been shown[6] that in intranasally infected rats alveolar cells filled with rickettsiae which desquamate are shed into the alveolar spaces and eliminated by natural ways. In no animal has any infected alveolar cell been seen in a blood vessel. The first three eventualities being thus dismissed as improbable, a weighty argument can be given in favor of the opinion that the reticular cells filled with rickettsiae found in the blood of mice and rats are reticuloendothelial cells, and more precisely, littoral cells of venous sinuses of the

7. Ricketts, H. T. and Wilder, R. M. 1910, J.A.M.A. **54**: 1373.

spleen. It seems highly probable, indeed, that cells like the one represented in figure 7, floating free in the lumen of the sinus, are eventually carried away by the blood stream and brought into the circulation, exactly in the same way as are brucella.[1] If this view is correct, it is readily admissible that many of the polymorphonuclear leucocytes and monocytes carrying rickettsiae, and a large number of the free organisms which were seen in the same sinuses of the spleen as the infected reticular cells and have been found in the blood, are of the same provenience. Thus, the mechanism of infection of the blood by rickettsiae would be as follows. In intranasally infected animals the organisms gain access to the circulation directly from the lymph spaces of the lung. After intraperitoneal inoculation, part of them find their way directly to the blood, while others strand in the organs of the abdominal cavity, but mainly in the spleen, where they are taken up by polymorphonuclear leucocytes, monocytes, adventitial cells of small blood vessels and littoral cells of the venous sinuses. Heavily infected littoral cells desquamate and are shed into the sinuses from which, together with the remaining infected material just described, they are brought into the circulation. Single rickettsiae may also pass into the circulation from heavily infected adventitial cells of small blood vessels. It appears, therefore, that the spleen plays an important role in the infection of the blood by rickettsiae.

The latter opinion does not imply, however, that the organisms cannot reach the blood also by other ways and from other organs. On the one hand, the presence of rickettsiae in the blood of splenectomized and intranasally infected mice shows that the spleen is not an indispensable link in the mechanism of infection of the blood by rickettsiae. On the other hand, although the organisms have not been found in the kidneys and in the liver of mice and rats, it is possible that the bone marrow of these animals, thus far not examined, is an important site of infection and, therefore, a potential source of spread of rickettsiae. Nevertheless, by its size, the large number of organisms it may contain and their close contact with the blood as is ensured in the spleen, this organ is considered to be one of the most important sources of infection of the blood by Rickettsia. In such circumstances, the virulence of the blood of animals infected with typhus is considered not to be due to any special affinity of Rickettsia to the blood but to be the consequence of the way in which this organism grows in the site of infection and in the spleen and of the pathological physiology of the latter. This view finds strong support in the experiments with dead rickettsiae, which have shown that the organisms are found in large numbers in the blood of mice injected intraperitoneally or intranasally with suspensions of formol-killed organisms.

The great differences in the incidence and the number of rickettsiae encountered in individual animals are very difficult to explain. All that can be said now in this respect is that they are not due to differences in the number of rickettsiae found in the lungs of intranasally infected mice and rats, as appears clearly from the observation of a series of 8 mice infected with classic typhus. In the blood of one of these animals rickettsiae were clearly more numerous than in that of the remaining mice, yet no striking differences in the rickettsial content of the lungs were noted in this series of animals.

From the standpoint of the defence of the animal against rickettsiae, it is important to understand the signifi-

cance of the intracellular position of the organisms. There is a fundamental difference between rickettsiae observed inside polymorphonuclear leucocytes and monocytes, on the one hand, and those found in reticular cells, on the other hand. Those in the former are considered to be phagocytized and doomed to destruction; those in the latter not only are not destroyed but bring about the death of the host cells. The opinion that inside phagocytes rickettsiae are finally destroyed finds support in the observation that, in striking contrast with reticular cells, parasitized leucocytes were invariably normal (fig. 3). The lack of signs of suffering on behalf of phagocytized rickettsiae observed in mice and rats is probably due to the short duration of the contact between the microbes and the leucocytes, which might have been not long enough to make apparent the effects of the digesting action of the phagocytes on the microbes.

An interesting finding is the large number of paired forms among the strikingly numerous rickettsiae observed in the blood of the two mice infected with classic typhus as mentioned previously. It shows that the proportion of the four types of rickettsiae, cocci, coccobacilli, paired forms and rods, is not fixed but changes. If, as is thought, paired rickettsiae are multiplication forms, the increase of their number might well be in connection with a particularly active multiplication of the organisms. This view finds support in the observation made in the lungs of rats, in which the short forms of rickettsiae, cocci and coccobacilli were more numerous in the earliest stages of the development of the organisms, while in later stages the long forms, diplobacilli and rods, were dominant.[6]

SUMMARY

1. Classic and murine rickettsiae morphologically similar to those grown in lungs, have been found in the blood of mice and rats infected intranasally or intraperitoneally, and organisms highly suggestive of rickettsiae have been encountered in the blood of a series of typhus patients.

2. There were great differences both in the incidence and in the number of rickettsiae in the blood between mice infected intranasally with classic or murine rickettsiae and between the latter and rats infected intranasally or irradiated and infected intraperitoneally with rickettsiae of murine type. Important differences in the number of the organisms encountered were noted also in the series of human patients examined.

3. The organisms were either free or inside polymorphonuclear leucocytes, monocytes and reticular cells. Those filling reticular cells bring about the death of the host cells; those inside phagocytes are eventually destroyed by the cells. Phagocytosis seems, therefore, to be an important factor in the defense of the organism against rickettsiae.

4. The infection of the blood by rickettsiae is not due to any special affinity of the organisms for the blood but is the consequence of the way in which they grow in the site of infection and in the organs of the reticuloendothelial system, mainly the spleen.

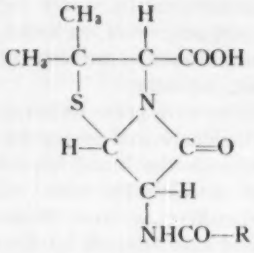
5. There is a striking similarity in the mechanism of infection of the blood by rickettsiae and brucellae.

2,3-DIMERCAPTOPROPANOL (BAL)–PENICILLIN COMPATIBILITY AND BRUCELLASTASIS IN VITRO

HARRY B. HARDING AND GORDON W. RALEIGH

From the Departments of Pathology and Medicine, Evanston Hospital, Evanston, Illinois, and the Department of Bacteriology, Northwestern University Medical School, Chicago, Illinois

According to Chain,[1] penicillin molecules are believed to have the general formula:



The radicle (R) may be one of the following groups which, when so conjugated, determines the penicillin:

$CH_3CH_2CH{=}CHCH_2{-}$
(Δ^2 pentenyl penicillin)

$CH_3CH{=}CHCH_2CH_2{-}$
(Δ^2 pentenyl penicillin)

$CH_3CH_2CH_2CH_2CH_2{-}$
(n-amyl penicillin)

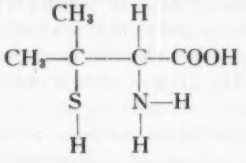
$CH_3(CH_2)_5CH_2{-}$
(n-heptyl penicillin)

$C_6H_5CH_2{-}$
(benzyl penicillin)

$HOC_6H_4CH_2{-}$
(*p*-hydroxybenzyl penicillin)

Among theories regarding the mechanism of bacteriostatic action of these substances, those stressing competitive enzyme inhibition find most favor. It is further postulated that the breakdown of penicillin yields a sulfhydril- (-SH) containing substance and that the sulfhydril groups compete for, and in effect bind, an essential enzyme of the bacterial protoplasm or its substrate. This is compatible with the general experience, cited by Chain,[1] that any of the penicillins can be broken down in vitro and possibly in vivo by the action of a number of substances (zinc, cadmium, lead, mercury and copper ions; primary alcohols and amines; ketonic reagents such as hydrazine and hydroxylamine; cysteine; bromine; oxidizing agents such as hydrogen peroxide and potassium permanganate; penicillinase) to yield:

a. penicillamine (thiol amino acid, β- thiovaline), which has the formula:

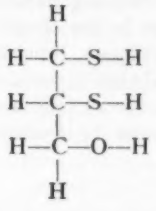


b. a penillo-aldehyde corresponding to one of the radicles (R) listed above

c. carbon dioxide

From these and numerous other observations, it appears reasonable to believe that the sulfhydril group of penicillamine may play an important role in the bacteriostatic action of penicillin.

2,3-Dimercaptopropanol (British Anti-Lewisite, BAL) has the formula:



Its effectiveness in certain heavy

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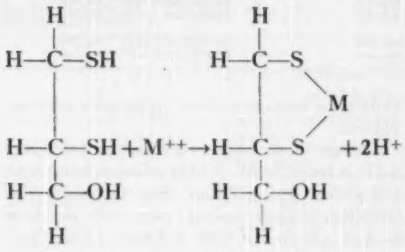
1. Chain, E. 1948, Endeavour, 7: 83–91.

metal intoxications is generally thought to be due to the action of its sulfhydril groups which bind the metal (M), possibly in accordance with the following general reaction (modified after Germuth and Eagle[2]):



If the pharamacologic importance of the sulfhydril groups common to penicillin and BAL be provisionally accepted, it becomes apparent that the two compounds may, through competition, be therapeutic antagonists. Furthermore, by combination with essential metallocatalysts, BAL might inhibit the bacteriostatic action of penicillin. Since penicillin is often a desirable adjunct of BAL therapy in heavy metal intoxications, clarification of this relationship appears necessary. Extension of the same reasoning indicates that BAL alone might be expected to have bacteriostatic properties. In order to test these possibilities, the following experiments were undertaken.

Experiment I.—This experiment was designed to determine whether BAL exerts any effect on penicillin, and if so, whether it increases or inhibits its activity.

A standard *Staphylococcus aureus* H strain and a known penicillin-sensitive strain of group A *Streptococcus pyogenes* designated 540/2 were the organisms employed for the test. A serial dilution method similar to those of Kolmer[3] and Smith et al[4] was used.

Bacterial suspensions for inoculation consisted of 1:1000 dilutions of 24-hour beef heart infusion broth cultures of *Staph. aureus* H and of *Str. pyogenes* 540/2.

One hundred mg of BAL* were measured to the bottom of a sterile 250 ml Erlenmeyer flask. To this were added 2 ml of 95% ethyl alcohol and the flask agitated for 20 to 30 seconds. Then 100 ml of warmed sterile beef heart infusion broth with pH of 7.0 were poured in slowly, using aseptic technique. This mixture constituted the initial BAL dilution of 1 mg per ml of broth.

Three series of test tubes, each set up in duplicate, were prepared: one with BAL alone, a second with BAL and penicillin, and a third with penicillin alone.

The first series consisted of two sets of 20 tubes each containing 5 ml of sterile beef heart infusion broth. To the first tube of the first set, 5 ml of the initial BAL dilution were added and the contents of the tube mixed four times. From the first tube 5 ml of the mixture were transferred to tube 2, and so on in series through tube 20, 5 ml being discarded from the last tube. A separate sterile 10 ml serologic pipette was used for each dilution. The second set of tubes was prepared in exactly the same manner.

A second series of tubes in duplicate sets was similarly prepared, using as the initial dilution a mixture in broth containing 1 mg BAL per ml and 0.05 mg per ml of standard penicillin.

For the third series, both sets of tubes were prepared in which the initial broth dilution contained only standard penicillin in 0.05 mg per ml amount.

After the dilutions had been made, to one set each of the three series, 1 drop of the *Staph. aureus* H suspension (see above) was inoculated into each tube, using a 1 ml sterile serologic pipette to transfer the material. The tubes of the other three sets were inoculated in the same manner with the *Str. pyogenes* 540/2 culture.

Appropriate controls were included and the entire lot incubated at 37 C for 24 hours.

At 24 hours the end point of beginning growth was read by observation. Gram-stained preparations were made where there was any doubt as to direct gross observation of growth. Table 1 gives the results of this experiment.

Experiment IIa.—Youmans and Youmans[5]

2. Germuth, F. G. and Eagle, H. 1948, J. Pharmacol. & Exper. Therap. 99: 397–410.
3. Kolmer, J. A. 1947, Penicillin Therapy, ed. 2, Appleton-Century Company, p. 32.
4. Smith, D. T., Martin, D. S., Conant, N. F., Beard, J. W., Taylor, G., John, H. I. and Poston, M. A. 1948, Zinsser's Textbook of Bacteriology, ed. 9, Appleton-Century Crofts Inc., p. 949.

* Obtained through the courtesy of Hynson, Westcott and Dunning, Inc., Baltimore, Md.

5. Youmans, G. P. and Youmans, A. S. 1949, Personal communication.

TABLE 1.—*Relation between BAL and penicillin.*

	Staph. aureus H	*Str. pyogenes* 540/2
Controls	Good growth Good growth	Good growth Good growth
BAL alone	No inhibition of growth No inhibition of growth	No inhibition of growth No inhibition of growth
Penicillin alone	Inhibition 6×10^{-4} mg/ml Inhibition 12×10^{-4} mg/ml	Inhibition 6×10^{-4} mg/ml Inhibition 3×10^{-4} mg/ml
BAL and penicillin	Inhibition 12×10^{-4} mg/ml Inhibition 24×10^{-4} mg/ml	Inhibition 6×10^{-4} mg/ml Inhibition 3×10^{-4} mg/ml

state that *Mycobacterium tuberculosis* var. *hominis* H37 is inhibited entirely by 1.25 mg% of BAL. No inhibition of the action of the drug for this organism was noted by them in 10 mg% concentration in 10% plasma. Because the effect of BAL on *Myco. tuberculosis* H37 was at variance with its effect on *Staph. aureus* H and *Str. pyogenes* 540/2, it was decided to determine the effect of BAL on a number of organisms. Consequently, BAL was tested against the organisms listed in table 2, employing the serial dilution method as outlined above.

Experiment IIb.—Since the results in table 2 show that both *Brucella abortus* strains were effectively inhibited by BAL, it was decided to test the effect of this drug on a number of different strains of *Brucella* organisms. The method employed was the one outlined in experiment I. Table 3 summarizes the results obtained using 6 different strains of *Brucella*.

Experiment IIc.—It was thought advisable to termine (a) whether plasma in 10% concentration would interfere significantly with the bacteriostatic effect of BAL on *Brucella*, (b) whether an inoculum 1000 times as strong would prevent inhibition by BAL, and (c) what change 8 days of incubation might have on the degree of inhibition by BAL of various strains of *Brucella*. As before, serial dilution methods were employed. Pooled sterile bovine plasma was used after first testing for the presence of agglutinins for *Br. abortus*. Only those batches without agglutinins were employed.

Change in the substrate medium from beef heart infusion broth to liver infusion broth with and without serum did not alter the results. The inhibition in each case was essentially the same as that indicated in table 4. Tables 4 and 5 present the results obtained in these experiments.

TABLE 2.—*Effect of BAL on various organisms.*

Organism	Hours of incubation at 37 C	Inhibiting concentration of BAL
Vibro comma (El Tor)	24	None
Alc. fecalis	24	0.125 mg/ml
Bacillus anthracis	24	None
Salmonella paratyphi	24	None
Pseudomonas aeruginosa	24	None
Klebsiella pneumoniae (Shinn)	24	None
S. pullorum	24	None
Shigella paradysenteriae Flexner	24	None
Br. abortus (Gibbs)	48	97×10^{-4} mg/ml*
Br. abortus 1163	48	97×10^{-4} mg/ml*

* End point not determined; 10 dilutions arbitrarily set up.

TABLE 3.—*Effect of BAL on different strains of* Brucella.

Organism	Hours of incubation at 37 C	Smallest inhibiting concentration of BAL
Br. suis 1753*	48	19×10^{-4} mg/ml
Br. abortus 1336*	48	24×10^{-4} mg/ml
Br. abortus 1163*	48	12×10^{-4} mg/ml
Br. melitensis 2493*	48	97×10^{-4} mg/ml
Br. abortus EHA	48	48×10^{-4} mg/ml
Br. abortus (Gibbs)	48	48×10^{-4} mg/ml

* Obtained through the courtesy of Dr. F. I. Huddleson, Michigan State College, East Lansing, Mich.

DISCUSSION

Numerous investigators[6–19] have indicated the importance of sulfhydril groups to certain enzymes of the bacterial and metazoan cell. The implications of such researches have recently

6. Barron, E. S. G. and Kalnitsky, S. 1944, Unpublished reports. Quoted by Herrmann, C. L., Nyman, B., Ogorzalik, S. and Chinn, H. I. 1948, Publication of the School of Aviation Medicine, Randolph Air Force Base, Texas.
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11. Hellerman, L., Chinard, F. P. and Deitz, V. R. 1943, J. Biol. Chem. **147:** 443–462.
12. Hopkins, F. G. and Morgan, E. J. 1938, Biochem. J. **32:** 611–620.

TABLE 4.—*Comparison of the bacteriostatic effect of BAL on various brucellae with and without the addition of 10% bovine plasma.*

Organism	Hours of incubation at 37 C	Inhibiting concentration without serum	Inhibiting concentration with serum
Br. suis 1753	48	19.5×10^{-5} mg/ml	19.5×10^{-5} mg/ml
Br. abortus 1336	48	48 $\times10^{-5}$ mg/ml	48 $\times10^{-5}$ mg/ml
Br. abortus 1163	48	48 $\times10^{-5}$ mg/ml	19.5×10^{-5} mg/ml
Br. melitensis 2493	48	19.5×10^{-5} mg/ml	19.5×10^{-5} mg/ml
Br. abortus EHA	48	19.5×10^{-5} mg/ml	19.5×10^{-5} mg/ml
Br. abortus (Gibbs)	48	19.5×10^{-5} mg/ml	19.5×10^{-5} mg/ml

TABLE 5.—*Effect of increased inoculum* on brucellastatic action of BAL at 48 hours and at 8 days.*

Organism	Hours of incubation at 37 C	Inhibiting concentration without serum	Inhibiting concentration with serum	Reincubation period	Inhibiting concentration without serum	Inhibiting concentration with serum
Br. suis 1753	48	7.8×10^{-4} mg/ml	7.8×10^{-4} mg/ml	8 days	15.6×10^{-3} mg/ml	15.6×10^{-3} mg/m
Br. abortus 1336	48	3.9×10^{-4} mg/ml	7.8×10^{-4} mg/ml	8 days	7.8×10^{-4} mg/ml	15.6×10^{-3} mg/m
Br. abortus 1163	48	3.9×10^{-4} mg/ml	3.9×10^{-4} mg/ml	8 days	7.8×10^{-4} mg/ml	7.8×10^{-4} mg/m
Br. melitensis 2493	48	3.9×10^{-4} mg/ml	3.9×10^{-4} mg/ml	8 days	7.8×10^{-4} mg/ml	7.8×10^{-4} mg/m
Br. abortus EHA	48	3.9×10^{-4} mg/ml	3.9×10^{-4} mg/ml	8 days	7.8×10^{-4} mg/ml	15.6×10^{-3} mg/m
Br. abortus (Gibbs)	48	7.8×10^{-4} mg/ml	7.8×10^{-4} mg/ml	8 days	15.6×10^{-3} mg/ml	15.6×10^{-3} mg/m

* One drop of a 48-hour Albimi broth culture per tube for each organism.

been emphasized by Smith[20] with respect to the glutathione oxidation-reduction potential system, which is based on sulfhydril-disulfide coversions. According to his concept a critical decrease in the number of active sulfhydril groups of this system, however produced, results in necrosis. That such may often be the nature of the initial biochemical lesion induced in the bacterial cell by various antibacterial agents, and in mammalian tissue cells by various living pathogenic agents and chemical intoxicants, is an attractive hypothesis rapidly gaining experimental support. On the other hand, little is known of the effect produced by addition of compounds having active sulfhydril groups to enzyme systems. Mac Cardle and Macnider[21] produced fatty change and parenchymal necrosis in the livers of dogs treated with BAL. However, the ultimate mechanism of these changes was not determined. Herrmann et al[8] cited unpublished experiments of Barron and Kalnitsky which indicated that certain heavy metals inactivate succinoxidase in vitro and that the inactivation can be reversed by BAL. The latter phenomenon is believed to be due to competition of the sulfhydril groups of BAL for the heavy metal, with consequent release of the enzyme sulfhydril groups from this bondage. A similar mechanism is held responsible for the efficacy of BAL in the treatment of certain heavy metal intoxications.[22] Webb and Van Heyningen[23] found BAL inhibited the metalloenzymes polyphenol oxidase (copper), carbonic anhydrase (zinc), catalase (iron), and peroxidase (iron). However, the metallo-

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21. MacCardle, R. C. and Macnider, W. D. 1947, Proc. Soc. Exper. Biol. & Med. **66**: 444–446.
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23. Webb, E. C. and Van Heyningen, R. 1947, Biochem. J. **41**: 74–78.

enzyme cytochrome oxidase was not affected by BAL. The apparently nonmetallic enzymes aldehyde mutase, phosphorylase, and glyoxalase were also inhibited. Since glutathione is thought to be an essential coenzyme of glyoxalase, it was postulated that BAL might compete through its sulfhydril groups for the activator site normally occupied by the sulfhydril group of glutathione. These investigators concluded that BAL probably generally inhibits enzymes having metal prosthetic groups with the exception of the cytochrome system. It is interesting to note that Pratt and Dufrenoy[24] have produced evidence that glutathione is involved in the mechanism of penicillin action in vitro. Thimann and Bonner[25] have indicated that plant growth is inhibited by coumarin and protoanemonin through reaction with sulfhydril enzyme and that this inhibition can be arrested by BAL. While the experimental evidence is not abundant, it is apparent that BAL or other exogenous substances having potentially active sulfhydril groups, such as penicillin, may inhibit enzyme systems by either binding metallic prosthetic groups, binding metal ion catalysts, or combining at sites normally reserved for the sulfhydril groups of coenzymes.

Table 1 presents the results of experiment I. It will be noted that BAL exerted no inhibitory effect on the growth of *Staph. aureus* H or *Str. pyogenes* 540/2. While marked inhibition was observed when penicillin was employed, the addition of BAL produced no significant alteration.

If the previously discussed postulates be accepted, it must be concluded that the chemical structure of BAL is such that its sulfhydril groups are unable to enter into the same reactions as, or compete with, the sulfhydril groups of penicillamine. Various investigators have determined the inhibition of certain bacteriostatic agents by sulfhydril compounds. Thus, inhibition of the bactericidal and viricidal effect of various heavy metals by BAL and other thio compounds is well known.[26–28] Cavallito and Bailey[29] demonstrated the cysteine inactivation of penicillin and a variety of less well known antibiotic substances. Cavallito[26] further demonstrated penicillin inactivation by cysteine esters, β-aminoethanethiol, and β-dimethylaminoethanethiol. The same investigator found poor inhibition by homocysteine, n-acetylcysteine, and thioglycollate. Chow and McKee[30] confirmed the penicillin inactivation by cysteine hydrochloride and felt that since simple mercaptans will not inactivate penicillin, other structural details than the mere presence of sulfhydril groups were involved. Denkelwater et al[31] found that streptomycin is inhibited by cysteine and 2-aminoethanethiol but not by thioglycollic acid, and similarly concluded that such inactivation was not a property of the sulfhydril groups alone. Simon[32] found that cysteine antagonizes the action of neoarsphenamine, penicillin, and streptomycin and felt that the inactivating reaction, through the sulfhydril group of cysteine,

24. Pratt, R. and Dufrenoy, J. 1948, J. Am. Chem. Soc. **70:** 161.
25. Thimann, K. V. and Bonner, W. D., Jr. 1949, Science, **109:** 444.
26. Cavallito, C. J. 1946, J. Biol. Chem. **164:** 29–34.
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30. Chow, B. F. and McKee, C. M. 1945, Proc. Soc. Exper. Biol. & Med. **58:** 175–177.
31. Denkelwater, R., Cook, M. A. and Tishler, M. 1945, Science, **102:** 12.
32. Simon, R. D. 1948, Brit. J. Exper. Path. **29:** 202–215.

occurred extracellularly. Schwartzman[33] noted the importance of disulfide linkages and possibly of sulfhydril groups to the antipenicillin potency of some amino acids. He indicated that the CH_3-S linkage might be associated with enhancement of penicillin effectiveness. Other sulfur-containing groups such as thiosulfate and metabisulfite were found by Leonard[34] to have no antipenicillin activity. The catalytic effect of metal ions on the alcoholysis of the penicillins has been shown by Chain et al[35] to be inhibited by BAL, and this is probably due merely to the metal binding property of BAL. The results of our experiment are compatible with the observations of Cavallito[26] that the ideal thiol inhibitor of penicillin should possess a basic amino group on the carbon atom adjacent to the carbon atom carrying the sulfhydril group and that the substituents on the nitrogen atom should be smaller than ethyl. From his observations one might have postulated the penicillin compatibility of BAL demonstrated by these experiments.

In experiment II the bacteriostatic property of BAL, with regard to a variety of organisms, was studied (table 2). As will be noted, there was a slight inhibition of the growth of *Alcaligenes fecalis* and marked inhibition of growth with two strains of *Br. abortus*. Both of these organisms ordinarily are penicillin-resistant. Extension of this work (table 3) revealed marked growth inhibition of one virulent and three laboratory strains of *Br. abortus* and one virulent strain of both *Br. suis* and *Br. melitensis*. Further investigation (tables 4 and 5) showed that the bacteriostatic effect of BAL, with regard to the above organisms, was not affected by the presence of bovine plasma in 10% concentration, increasing the inoculum 1000-fold, maintenance of incubation for 8 days, or change of substrate to the more favorable liver infusion broth. It is felt that these data conclusively demonstrate in vitro brucellastasis by BAL.

That sulfhydril compounds should have antibacterial action under certain circumstances seems reasonable in the light of previous theoretical considerations. Volkert and Horsfall[28] were able to decrease the virulence and hemagglutinating property of the pneumonia virus of mice with glutathione and concentrated lung tissue. In each instance the effect was attributed to the sulfhydril groups present. Miller and Elson[36] employed salts of dithiocarbamic acid derivatives with some antibacterial effectiveness. Goth and Robinson[37] demonstrated similar effectiveness against a variety of bacteria using a series of dithiocarbamates and their bismuth derivatives. In these instances the possible role of potential sulfhydril groups is not clear. Goth and Robinson[37] found *Br. melitensis*, among other organisms, to be inhibited and suggested that this effect was due to interference with metal catalyzed enzyme reactions. D'Alessandro and Comes[38] were able to inhibit the growth of *Br. melitensis*, *Br. abortus*, and *Br. suis*, as well as a variety of other organisms, with *p*-sulfonamidethiophenol and thiophenal. This action was antagonized by thioglycollate, glutathione, and cysteine. The authors concluded that the bacteriostatic action of the thio compounds was related to the sulfhydril groups of bacteria.

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34. Leonard, C. S. 1946, Science, **104**: 501–502.
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37. Goth, A. and Robinson, F. J. 1948, J. Pharmacol. & Exper. Therap. **93**: 430–433.
38. D'Alessandro, G. and Comes, R. 1947, Boll. Soc. Ital. biol. sper. **23**: 1092.

Youmans and Youmans[5] have demonstrated a tuberculostatic effect in vitro by BAL in concentrations as low as 1.25 mg%. Klein and Perez[37] were unable to find any inhibition of influenza A virus by BAL. However, Anderson and Chin[39] found BAL to be synergistic with subtilin in the bacteriostasis of a number of mycobacteria and to potentiate the action of streptomycin against a pathogenic strain. These investigators found BAL to inhibit the streptomycin activity against *Micrococcus lysodeikticus*. Finally, they found BAL to be tuberculostatic in a concentration of 1:10,000.

Our results indicate a clear-cut brucellastatic effect by BAL in vitro. While the mechanism is unknown it appears reasonable to believe that the active sulfhydril groups of BAL play an important part by inactivating metallic ion catalysts or metalloenzymes or by competing for sulfhydril sites normally taken by coenzymes. In favor of the first possibility is the observation of McCullough et al[40] that magnesium is essential for the growth of certain strains of *Br. suis*. While Gerhardt and Wilson[41] have reemphasized the possibility that glycerol may function in ways other than as a primary nutrient in the metabolism of brucellae, and the chemical similarity of BAL and glycerol is apparent, the possible significance of this relationship is obscure. It is interesting to note that Broschard et al[42] apparently did not find sulfur in their chemical analysis of aureomycin, the most effective in vivo brucellastatic agent developed to date. It therefore seems unlikely that aureomycin and BAL operate ,in this respect, by a common mechanism. Such a possibility may prove advantageous in combined therapy if experiments now in progress prove BAL an effective brucellastatic drug in vivo.

39. Anderson, H. H. and Chin, Y. 1947, Science, **106**: 643–644.
40. McCullough, W. G., Mills, R. C., Herbst, E. J., Roessler, W. G. and Brewer, C. R. 1947, J. Bact. **53**: 5–15.
41. Gerhardt, P. and Wilson, J. B. 1948, J. Bact. **56**: 17–24.

SUMMARY AND CONCLUSIONS

1. The chemistry and acceptable theories regarding the mode of operation of penicillin and 2,3-dimercaptopropanol (BAL) are reviewed.

2. The necessity of determining the compatibility of these compounds is indicated.

3. The literature is reviewed and theoretical considerations regarding the importance and role of sulfhydril enzymes and exogenous sulfhydril compounds are discussed.

4. 2,3-Dimercaptopropanol (BAL) exerts no effect on the bacteriostatic action of penicillin under the conditions of these experiments.

5. 2,3-Dimercaptopropanol (BAL) is an effective brucellastatic agent under the conditions of these experiments.

42. Broschard, R. W., Dornbush, A. C., Gordon, S., Hutchings, B. L., Kohler, A. R., Krupka, G., Kushner, S., Lefemine, D. V. and Pidacks C. 1949, Science, **109**: 199–200.

A FURTHER INVESTIGATION OF THE RESPONSE OF *LEUCONOSTOC* TO NICOTINIC ACID AND RELATED COMPOUNDS

STEWART A. KOSER AND GEORGE J. KASAI

From the Department of Bacteriology and Parasitology and the Walter G. Zoller Memorial Dental Clinic, The University of Chicago, Chicago 37, Illinois

This report is an extension of an earlier study which dealt with certain unusual aspects of the growth response of *Leuconostoc* to nicotinic acid and related compounds. It was first reported by Johnson[1] that *Leuconostoc mesenteroides* 9135, though requiring nicotinic acid, did not respond to ordinary amounts of nicotinamide or nicotinuric acid. It was found by the writers[2] that of 6 *Leuconostoc* cultures which needed nicotinic acid for prompt growth in a casein digest medium, two of them failed to grow in the presence of nicotinamide, diphosphopyridine nucleotide (DPN), or triphosphopyridine nucleotide (TPN), while a third culture failed to grow in nicotinamide but grew promptly when DPN or TPN was supplied. Also, these 3 cultures did not respond to ordinary amounts of nicotinuric acid and methyl nicotinate, compounds which usually are utilized readily by other nicotinic acid requiring bacteria.

The present work deals with certain aspects of the behavior of *Leuconostoc* not covered in our earlier report and includes also some tests with *Streptococcus salivarius*. The series of *Leuconostoc* cultures was extended to include additional representatives of the genus. A synthetic medium of amino acids and other definite compounds was used for this larger series of cultures in place of the casein hydrolyzate medium previously employed. It was found that apparent inability to make use of ordinary vitamin amounts of nicotinamide is common to a number of *Leuconostoc* group organisms and that this may or may not be coupled with inability to grow in the presence of DPN. A number of other experiments dealing with the effect of surface active agents, "adaptation" through successive transfers, methylene blue reduction tests, and the response of *Pasteurella* cultures to the products of *Leuconostoc* synthesis are included.

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1. Johnson, B. C. 1945. J. Biol. Chem. **159**: 227.
2. Koser, S. A. and Kasai, G. J. 1948, J. Infect. Dis. **83**: 271.

METHODS

Basal mediums.—The amino acid medium employed in the present study was patterned after that used by Hac et al[3,4] for assay of glutamic and aspartic acids by the use of lactobacilli and *Leuconostoc*. A few minor changes were made in the content of the medium: norvaline, choline, and inositol were omitted; the amount of glutamic acid was reduced from 1000 to 400 μg per ml; and thymine was added. The composition of our medium per liter of redistilled water then was: 100 mg of each of L-arginine hydrochloride, L-cystine, glycine, L-histidine hydrochloride, L-hydroxyproline, L-leucine, L-lysine hydrochloride, L-proline, L-tryptophane, and L-tyrosine; 200 mg of each of DL-alanine, DL-isoleucine, DL-methionine, DL-norleucine, DL-phenylalanine, DL-serine, DL-threonine, and DL-valine; 400 mg of each of L-aspartic acid and L-glutamic acid; 10 mg of each of adenine sulfate, guanine hydrochloride, thymine, uracil, and xanthine; 1.0 g K_2HPO_4, 1.0 g KH_2PO_4, 200 mg $MgSO_4 \cdot 7H_2O$, 10 mg NaCl, 10 mg $FeSO_4 \cdot 7H_2O$, 7.5 mg $MnCl_2 \cdot 4H_2O$, 6 g sodium acetate, 3 g $(NH_4)_2SO_4$, 10 g glucose, 10 mg pyridoxine hydrochloride, 300 μg *p*-aminobenzoic acid, 200 μg calcium pantothenate, 200 μg riboflavin, 200 μg

3. Hac, L. R., Snell, E. E. and Williams, R. J. 1945, J. Biol. Chem. **159**: 273.
4. Hac, L. R. and Snell, E. E. 1945, J. Biol. Chem. **159**: 291.

thiamine hydrochloride, 10 μg pteroylglutamic acid, and 1.0 μg biotin.

The medium was adjusted to pH 6.8 with sodium hydroxide, filtered, dispensed in 10 ml amounts in standardized 18×150 mm tubes, and autoclaved at 10 pounds extra pressure for 20 minutes. Nicotinic acid, nicotinamide, or DPN was added separately to individual tubes as called for in different experiments. Stock solutions of nicotinamide and the coenzyme were sterilized by filtration through sintered glass filters.

A casein digest medium was used for some experiments. This was similar to that previously employed[2] and included 100 μg pteroylglutamic acid per liter since some *Leuconostoc* strains either need this compound or grow more readily when it is supplied. This medium was also adjusted to pH 6.8 and filtered. As in the preceding medium, small amounts of some of the salts are lost in the process of preparation because a light haziness appears when the medium is heated, necessitating filtration. By omitting the iron and manganese this light precipitate could be avoided, but inclusion of these salts seemed preferable from the standpoint of growth response, even though uncertain quantities of them are left in solution in the medium.

Cultures.—Several representatives of each of the three species of *Leuconostoc* were secured from the American Type Culture Collection. For additional *Leuconostoc* cultures the writer is indebted to Dr. C. S. McCleskey of Louisiana State University, Baton Rouge, and to Dr. C. F. Niven of the American Meat Institute Foundation of Chicago.

The *Str. salivarius* cultures were isolated from the human mouth by streaking swabs on sucrose-meat infusion-yeast extract agar plates, selecting the gum-producing colonies, and subjecting them to replating and reisolation to secure pure cultures. These organisms were included in the study because of certain similarities to *Leuconostoc* in polysaccharide synthesis from sucrose. All *Str. salivarius* strains used in this study were secured from different individuals. The gram-positive cells were usually arranged in short chains. Cultures on blood agar plates did not produce hemolysis. In litmus milk they produced an acid curd with reduction but no peptonization. All cultures produced acid promptly in glucose, lactose, and sucrose broths, and did not grow at 50 C.

Inoculum.—The inoculum received by each tube in the tests consisted usually of 0.001 ml of a 20- to 24-hour culture which had been grown in the casein digest medium with a small amount of nicotinic acid. The 0.001 ml was obtained by dilution of the culture in the basal medium.

Incubation and measurement of growth.—The temperature of incubation was 30 C for *Leuconostoc* and 37 C for *Str. salivarius*. The growth response was measured by turbidity with a Lumetron colorimeter, using a 420 mμ filter and an uninoculated tube of medium set at 100 as the blank. Readings were made daily for the first 3 or 4 days, with one or two additional readings at 6 or 7 days or occasionally later. In stating turbidity in the tables it seemed more satisfactory to consider a clear tube as 0 rather than 100. Thus the colorimeter figures have been reversed with respect to 100, that is, a reading on the Lumetron of 97 is given as 3, 75 as 25, 31 as 69, and so on. This has the advantage of showing no visible growth as 0, light growth by small numbers, and increasingly heavier growth by larger numbers.

RESULTS

Growth response of the organisms in the synthetic medium in the presence of either nicotinic acid, nicotinamide, or DPN is shown in table 1. In the basal medium a light turbidity appeared which is probably the result of traces of impurities in the compounds used in the medium together with a small amount of carry-over of nicotinic acid in the inoculum. Two of the strains, 8042 and 8082, are not absolutely dependent upon nicotinic acid and grow slowly in the basal medium without it. All cultures, with one exception, responded readily to nicotinic acid, growing promptly and producing heavy turbidity within 24 or 48 hours. The one exception, *L. citrovorum* 8081, failed to grow in the basal medium even when supplemented with all crystalline vitamins available. This organism evidently has additional requirements which have been studied recently by Sauberlich and Baumann[5] and Steele et al.[6]

The peculiarity of lack of response to nicotinamide in ordinary vitamin amounts is evidently rather common in the *Leuconostoc* group, for it is exhibited

5. Sauberlich, H. E. and Baumann, C. A. 1948, J. Biol. Chem. **176**: 165.
6. Steele, B. F., Sauberlich, H. E., Reynolds, M. S. and Baumann, C. A. 1949, J. Biol. Chem. **177**: 533.

TABLE 1.—*Comparative growth in synthetic medium in presence of nicotinic acid, nicotinamide, and diphosphopyridine nucleotide.*

Organism		Basal medium	Basal medium, plus 10^{-6} *M*		
			Nicotinic acid	nicotinamide	DPN
		Turbidity readings at 3 days			
L. mesenteroides	8042	14*	62	60	60
L. mesenteroides	8293	7	87	**7**	**9**
L. mesenteroides	8357	10	82	82	83
L. mesenteroides	9135	8	84	**11**	**85**
L. mesenteroides	D11	15	88	89	89
L. mesenteroides	A152	6	85	**8**	**86**
L. mesenteroides	B700	3	73	**5**	**73**
L. mesenteroides	F711	8	85	86	87
L. dextranicum	8086	15	86	**18**	**16**
L. dextranicum	8358	7	87	**11**	**9**
L. dextranicum	8359	6	85	**8**	**9**
L. citrovorum	7013	15	66	65	65
L. citrovorum	8081	1	1	1	0
L. citrovorum	8082	29*	59	59	60
Leuconostoc-like	M3A	7	86	86	81
Leuconostoc-like	S26A	4	80	67	61
Str. salivarius	21 strains	5 to 12	67 to 88	61 to 89	66 to 90

The basal medium in this case is the amino acid synthetic medium plus all growth factors with the exception of nicotinic acid or related compounds.

In the figures showing turbidity, 0 represents complete light transmission as compared with an uninoculated tube of the medium. Increasing turbidities are shown by higher figures, with 60 to 80 representing heavy growth.

Significant results in the nicotinamide and DPN columns, as compared with nicotinic acid, are shown in boldface. The DPN used here was 60% pure.

* Slow growth without the vitamin, becoming gradually heavier after 3 days, was shown by these 2 cultures.

by 7 of the 15 strains which grew in the presence of nicotinic acid: numbers 8293, 9135, A152, B700, 8086, 8358, and 8359. In the case of 4 of these cultures it is coupled with failure to grow in the presence of the coenzyme; the remaining 3, while failing to respond to nicotinamide, grow in coenzyme as promptly and as luxuriantly as in the presence of nicotinic acid. The failure to respond to nicotinamide or coenzyme is quite definite. The light turbidity appearing in such tubes is of the same order as that in the control tubes of basal medium without the vitamin. These tubes were routinely incubated for 7 days and at times for longer periods without obtaining any evidence of definite growth in 1.0 μg per ml amounts or corresponding molar amounts of the coenzyme.

In additional experiments larger amounts of nicotinamide, 36 μg per ml, were supplied in the synthetic medium. Cultures which failed to respond to ordinary amounts of the amide exhibited growth at 30 C in the presence of the larger amount, but turbidity did not become as heavy as that in the presence of 1.0 μg nicotinic acid. In this respect and also in regard to the results shown in table 1, the present results with the amino acid synthetic medium confirm and extend previous findings[2] with a smaller number of cultures in a casein digest basal medium.

Effect of surface-active agents and other changes in the basal medium.—The possibility was considered that such agents or changes might influence response of the *Leuconostoc* cultures which previously had failed to grow in the presence of ordinary vitamin amounts of nicotinamide. Varying amounts of Tweens 20, 40, 60, and 80, sodium ricinoleate, and sodium lauryl sulphate were incorporated in separate experiments in the casein hydrolyzate basal medium. Tween 20 contains laurate, Tween 40 palmitate, Tween 60 stearate, and Tween 80 oleate. The Tweens were used in amounts of 10, 100, and 1000 μg per ml; sodium ricinoleate in amounts of 0.1, 1.0, and 10 μg per ml; and sodium lauryl sulphate in amounts of 0.3, 1.0, and 3.0 μg per ml. Larger quantities of the last two compounds were distinctly inhibitive and were not employed. The medium contained an ample supply of biotin and other growth factors with the exception of nicotinic acid. Nicotina-

mide was present in the amount of 1.0 μg per ml. None of these compounds in any of the concentrations employed produced any definite effect in accelerating growth of the *Leuconostoc* cultures which ordinarily failed to grow in the presence of nicotinamide.

The addition of 25 μg and 100 μg of indole-3-acetic acid, in the presence of 1.0 μg per ml of nicotinamide, produced no detectable increase in growth of the 7 cultures which failed to respond to nicotinamide. This substance was used because of the recent report of Galston and Hand[7] that the compound, although itself without effect, enhanced growth (acid production) of *Lactobacillus arabinosus* 17–5 in the presence of nicotinamide.

Other changes in the basal medium also were tried with similar negative results with respect to growth in nicotinamide. These included, in separate experiments, an increase in the glucose concentration to 2% , an increase in the sodium chloride to 1.4% and to 3%, an increase in the amino acids to 4 times the usual amount, and several combinations of the foregoing items, and also a decrease in glucose concentration to 0.3% coupled with omission of acetate. A similar lack of response to nicotinamide following changes in the initial pH of the basal medium from 5.6 to 7.3 and to combinations of nicotinamide and nicotinic acid was noted in our earlier report.[2]

Methylene blue reduction.—The comparative response of two of the *Leuconostoc* cultures to nicotinic acid, nicotinamide, and diphosphopyridine nucleotide was studied also by means of methylene blue reduction. In the first experiments of this nature considerable irregularity was encountered and results were not always consistent. In general the Thunberg tube containing nicotinic acid showed speedier reduction than the others, but results could not always be duplicated and at times the control tubes showed reduction of the dye as speedily as any of the others. After a number of trials with different amounts of glucose, vitamin, methylene blue, and varying pH levels of the phosphate buffer, a combination of materials was found which gave more consistent results. It was noted, too, that the activity of the cell suspension was more vigorous when the time elapsed in its preparation and before its use in the test was kept at a minimum.

To secure cells for the tests *Leuconostoc* cultures were grown in casein digest basal medium with 1.0 % glucose, the necessary supplements, and 0.005 μg per ml of nicotinic acid. This amount of vitamin is distinctly suboptimum but supports development of a crop of cells sufficient for the tests. Cells from 22- to 24-hour cultures incubated at 30 C were centrifuged, washed twice with *M*/30 phosphate buffer pH 6.8, and suspended in the same buffer solution. The volume used to take up the cells was adjusted to give a turbidity reading on the Lumetron colorimeter of 20 to 25, or in some of the tests of 15 to 17, with the 420 mμ blue filter.

Comparative data are given in table 2 for two of the cultures which failed to respond to nicotinamide and DPN in growth tests. The figures shown are typical of other tests with the particular combination of reagents used. Nicotinic acid produced a distinct stimulation of metabolism, while the effect of nicotinamide and DPN was much less marked or, in some tests, essentially the same as the control tube without the vitamin.

Adaptation to nicotinamide.—Some experiments were undertaken to determine whether variants capable of growth in ordinary vitamin amounts of nicotinamide could be obtained from stock *Leuconostoc* cultures which did not show

7. Galston, A. W. and Hand, M. E. 1949, J. Biol. Chem. **178**: 967.

TABLE 2.—*Effect of nicotinic acid, nicotinamide, and diphosphopyridine nucleotide on methylene blue reduction.*

	Time (in minutes) required for reduction	
	Leuconostoc 8086*	Leuconostoc 8293*
Control	45	51
Nicotinic acid	25	20
Nicotinamide	44	42
DPN	47	42

Each Thunberg tube received 0.3 ml of 2% glucose and 1.0 ml of 1:10,000 methylene blue solution. The last three tubes received 15 μg of nicotinic acid, nicotinamide, or 80 μg of DPN, respectively. After evacuation of the tubes and adjustment to 30 C, 1.0 ml of cell suspension in *M*/30 phosphate buffer at pH 6.8 was tipped in. The glucose, methylene blue, and vitamin solutions were all dissolved in the same phosphate buffer solution. The total volume was 3.0 ml and the tubes were held at 30 C.

* These two cultures failed to grow in the presence of nicotinamide and DPN.

this property. The ability of *Leuconostoc* to grow in the presence of higher concentrations of nicotinamide at 30 C offered the possibility of securing cell multiplication through successive transfers in the presence of nicotinamide and of a gradual reduction in the amount of vitamin as the series progressed.

Several series of transfers were attempted, though not all were productive of variants capable of growth in the presence of small amounts of nicotinamide. From a total of 13 such series in which cultures 8293, 9135, A152, B700, 8086, 8358, and 8359 were used, 6 variants were obtained capable of continuous good growth in successive transfers in the presence of 1.0 μg nocotinamide per ml of medium. These 6 variants were obtained mostly after 20 to 35 transfers during which the concentration of nicotinamide was decreased from an initial amount of 36 μg per ml to 18 μg and then to 9 or 10, 5, and so on to 1.0 μg per ml. In one instance only 15 transfers were required. In other cases, however, it was impossible to secure such variants even though the series involved over 50 successive transfers and 4 months time. The casein digest basal medium was used for all of these experiments with incubation at 30 C. Transfers from one tube to another in the series were made with a wire loop of about 3 mm diameter.

After variants were obtained several of them were tested in graded amounts of nicotinamide, DPN, methyl nicotinate, and nicotinuric acid to compare the growth response to that in nicotinic acid. The results of two such tests are shown in tables 3 and 4. The behavior of the original stock cultures (8293 and A152) from which these variants were obtained has been given in table 1. Growth of the 8293 variant (table 3) in amounts of nicotinamide below 3×10^{-6} *M* was distinctly inferior to that supported by comparable quantities of nicotinic acid, both in rate and in final turbidity attained. In turn, the response to DPN was markedly slower and inferior than to nicotinamide. Only the two largest amounts of DPN (60% pure) supported slow growth of the organism; these molar concentrations correspond approximately to 6.6 and 2.0 μg per ml. Thus a distinct lowering of effectiveness for the 8293 variant is seen in the order of nicotinic acid > nicotinamide > co-

TABLE 3.—*Growth of the 8293 variant in nicotinic acid, nicotinamide, and related compounds.*

Molar concentration	Nicotinic acid	Nicotinamide		DPN		Methyl nicotinate		Nicotinuric acid
	72 hr.	72 hr.	7 days	72 hr.	7 days	72 hr.	7 days†	10 days
1×10^{-5}	81	80	83	12	30*	12	22	3
3×10^{-6}	81	60	76	6	20*	8	10	2
1×10^{-6}	77	10	41	0	3	4	8	2
3×10^{-7}	53	7	25	0	3	0	5	2
1×10^{-7}	24	0	5	0	2	0	3	2
3×10^{-8}	14	0	3					
1×10^{-8}	8	0	2					
None	0	0	3	0	2	0	2	3

Casein hydrolyzate basal medium plus all supplements except nicotinic acid; 30 C incubation. DPN was 60% pure.

* Somewhat heavier growth at 10 days in the two largest amounts of DPN.

† Heavier growth not apparent after 7 days.

enzyme. The relative ineffectiveness of DPN in relation to nicotinamide in this instance is the reverse of that seen in several of the *Leuconostoc* stock cultures (9135, A152, B700) which grew promptly in the presence of DPN but not in nicotinamide.

Methyl nicotinate and nicotinuric acid were included in the tests since these compounds in ordinary amounts did not support growth of several *Leuconostoc* stock cultures,[2] though other nicotinic acid requiring bacteria make use of them readily. The response of variant 8293 to methyl nicotinate was quite feeble, while no response to nicotinuric acid could be detected. Thus the ability of the variant to grow in approximately 1.0 μg per ml nicotinamide was not accompanied by a similar response to methyl nicotinate or nicotinuric acid.

The A152 variant (table 4) came from a stock culture which originally grew readily in 10^{-5} *M* amounts of nicotinic acid and DPN but not of nicotinamide. After 15 successive transfers with decreasing amounts of the amide to a figure of 1.0 μg per ml, and then 3 additional transfers in the 1.0 μg per ml amount of nicotinamide, the culture was tested against the compounds as shown in table 4. The response to smaller quantities of the amide is distinctly inferior to that of comparable concentrations of nicotinic acid. The response to DPN is vigorous, as it was in the original culture. Methyl nicotinate in the largest amounts used produced only a moderate increase in growth over that of the control tubes, while nicotinuric acid did not support growth. Thus in the A152 variant we now have a culture which grows well in 10^{-5} *M* amounts of nicotinic acid, nicotinamide, or DPN but grows poorly in methyl nicotinate and not in nicotinuric acid.

Hemophilus factor.—Leuconostoc cultures which fail to grow in nicotinamide or in DPN apparently synthesize DPN or a substance which supports growth of *Hemophilus parainfluenzae*[2] when grown in nicotinic acid. One of the questions arising from this observation concerns the nature of the hemophilus growth factor formed by *Leuconostoc* and whether it contains nicotinamide. An attempt was made to clarify this point by taking advantage of the known growth requirements of *Hemophilus* and of some members of the *Pasteurella* group. Some *Pasteurella* types, such as *Past. avicida*, *Past. boviseptica*, *Past. suiseptica* and others fail to respond to nicotinic acid but grow readily in the presence of nicotinamide.[8,9] In this respect their behavior is the reverse of that of certain *Leuconostoc* cultures. Our object was to determine whether the hemophilus growth factor formed by

TABLE 4.—*Growth of the A152 variant in nicotinic acid, nicotinamide, and related compounds.*

Molar concentration	Nicotinic acid	Nicotinamide		DPN	Methyl nicotinate	Nicotinuric acid
	72 hr.	72 hr.	6 days	72 hr.	72 hr.	6 days
1×10^{-5}	84	76	80	81	27	7
3×10^{-6}	84	61	77	68	17	6
1×10^{-6}	75	15	51	48	11	5
3×10^{-7}	52	6	4	31	9	4
1×10^{-7}	31	6	3	19	7	4
3×10^{-8}	15	6	3	12	6	4
1×10^{-8}	8			9	6	4
None	5	5	4	5	5	3

Casein hydrolyzate basal medium plus all supplements except nicotinic acid; 30 C incubation. DPN was 60% pure. The A152 stock culture grew in DPN but not in nicotinamide.
All observations were continued through 9 or 10 days. In DPN, methyl nicotinate, and nicotinuric acid there was no increase in growth after the incubation times shown above.

8. Koser, S. A., Berkman, S. and Dorfman, A. 1941, Proc. Soc. Exper. Biol. & Med. **47**: 504.
9. Berkman, S. 1942, J. Infect. Dis. **71**: 201.

Leuconostoc, after its breakdown by relatively mild treatment sufficient to destroy its activity for *Hemophilus*, would support growth of *Pasteurella*.

To secure the hemophilus factor *Leuconostoc* cultures 8086, 8293, and 9135 were grown in casein hydrolyzate medium with 1.0 μg nicotinic acid per ml. After 48 hours incubation at 30 C the cultures were adjusted to approximately pH 6.9 with sterile 1*N* sodium hydroxide and heated at 80 C in a water bath for 15 minutes. Sterility tests were made to be sure *Leuconostoc* cells were killed. The heated cultures were centrifuged to throw down the cells and the supernatant removed. Doubtless much of the factor formed by *Leuconostoc* adheres to the cells and is lost, so that our tests on the supernatant do not in any sense represent an assay of the substance. After centrifuging each supernatant was divided into two portions; one was used as such and the other was heated in the autoclave at 15 pounds pressure for 30 minutes before use in subsequent growth tests.

For growth tests with *H. parainfluenzae* a veal infusion broth with 0.5% glucose was used as the basal medium. The *Hemophilus* grew in this medium when supplemented with small amounts of a known DPN preparation.

For tests with *Pasteurella* a casein hydrolyzate-glucose-salts medium supplemented with cystine, tryptophane, biotin, and calcium pantothenate was used as the basal medium. Several *Pasteurella* cultures of the animal hemorrhagic septicaemia types were obtained. In the foregoing medium all of them grew in the presence of 0.5 μg per ml nicotinamide, but not with nicotinic acid, nor in the basal medium without nicotinamide.

In tests the autoclaved and unautoclaved supernatants from the *Leuconostoc* cultures were added in several different amounts to tubes of the basal mediums for *Hemophilus* and *Pasteurella*. These tubes were then given small inoculums of either *Hemophilus* or *Pasteurella* cells, incubated at 37 C, and the amount of growth estimated at several intervals. A typical result is given in table 5 for supernatants from cultures 8086 and 8293. Similar results were secured with *Leuconostoc* 9135.

Unautoclaved supernatants from the two *Leuconostoc* cultures supported growth of both *Hemophilus* and *Pasteurella*, which would be expected if the substance formed by *Leuconostoc* is DPN or something physiologically equivalent. The *Pasteurella* types respond to DPN as well as to nicotinamide, as is known from previous work. However, a different result was obtained with the autoclaved supernatants. This treatment destroyed activity for *Hemophilus*, while the growth-promoting effect for *Pasteurella* was retained. The growth of *Pasteurella* in this case evidently is not due to the hemophilus factor, since this has been broken down or inactivated by the autoclaving. Also, *Pasteurella* does not respond to nicotinic acid. While this suggests that a coenzyme containing nicotinamide is formed by *Leuconostoc* cultures, such a conclusion must be regarded with considerable reservation. This is necessarily so because of our incomplete knowledge of the activity of substances related to nicotinamide and DPN (some perhaps now unknown), and of the activity of various possible breakdown products of DPN. Though suggestive only, the findings may be of some interest in the question of the constitution of the *Hemophilus*-active substance formed by *Leuconostoc* cultures which fail to respond to nicotinamide or DPN.

SUMMARY

The peculiarity of lack of response to ordinary amounts of nicotinamide or to diphosphopyridine nucleotide, coupled

TABLE 5.—*Showing response of* Hemophilus *and* Pasteurella *to* Leuconostoc *supernatant.*

Leuconostoc supernatant after growth in casein digest with nicotinic acid		Growth at 48 hours		
Culture	Ml of supernatant added per 10 ml tube	*H. parainfluenzae* 429	*Past. boviseptica* 53	*Past. suiseptica* 221
8086	1.0	56		
	0.3	51	30	29
	0.1	7	15	15
	0.03	0		
8293	1.0	57		
	0.3	56	44	41
	0.1	31	26	28
	0.03	0		
8086 autoclaved	1.0	0		
	0.3	0	39	38
	0.1	0	27	27
	0.03	0	9	8
	0.01	0	3	4
8293 autoclaved	1.0	0		
	0.3	0	42	42
	0.1	0	39	39
	0.03	0	15	14
	0.01	0	5	6
Controls				
a—Glucose veal infusion broth		0		
b—a plus coenzyme I, 6.6 μg/ml		55		
c—b autoclaved		0		
d—Basal medium for *Pasteurella*			0	2
e—d plus nicotinamide, 0.5 μg/ml			32	30
f—d plus nicotinic acid, 0.5 μg/ml			2	3

with a nicotinic acid requirement, is evidently rather common among organisms of the *Leuconostoc* group, for it was encountered in 7 of a total of 15 cultures which grew in a synthetic medium plus nicotinic acid. Four of the 7 *Leuconostoc* strains showed no growth response to either nicotinamide or DPN, and the 3 others failed to grow in the presence of nicotinamide but grew in DPN as promptly and vigorously as in nicotinic acid. The remaining 8 *Leuconostoc* strains responded in a more conventional manner to nicotinic acid, nicotinamide, and DPN.

Twenty-one strains of *Streptococcus salivarius*, another gum-producing coccus, all exhibited a prompt growth response to nicotinic acid, nicotinamide, or DPN.

With *Leuconostoc* cultures which failed to respond to nicotinamide, agents such as the Tweens, sodium ricinoleate, and sodium lauryl sulphate did not facilitate growth in the presence of nicotinamide. The presence of indole-3-acetic acid, and increases in the amounts of amino acids, glucose, or sodium chloride in the basal medium, or of several combinations of such additions, likewise failed to produce any notable difference in response to nicotinamide.

In methylene blue reduction tests nicotinic acid produced a distinct stimulation of metabolism, while the effect of nicotinamide and DPN was much less marked.

Variants "adapted" to growth in 1.0 μg per ml amounts of nicotinamide may be obtained by successive transfers starting with large amounts of nicotinamide, 36 μg per ml and gradually reducing the concentration to 1.0 μg per ml. Quantitative differences in the response of such variants to nicotinamide, DPN, methyl nicotinate, and nicotinuric acid were observed.

The hemophilus growth factor formed by *Leuconostoc* in the presence of nicotinic acid, when inactivated for *Hemophilus* by autoclaving, supported growth of *Pasteurella* cultures which are not stimulated by nicotinic acid.

THE RECOVERY OF POLIOMYELITIS VIRUS AFTER PARENTERAL INTRODUCTION INTO COCKROACHES AND HOUSEFLIES

HERBERT S. HURLBUT

From the Naval Medical Research Institute, Bethesda, Maryland

Poliomyelitis virus has been isolated from filth-frequenting flies caught in nature during several epidemics in recent years.[1–3] When the Lansing strain of the virus was fed to house flies under experimental conditions, virus was not recovered after 48 hours.[4,5] Recently Melnick and Penner[6] fed virus in human stools to the blowfly *Phormia regina* and were able to recover it from the flies after about two weeks, and from the fly excreta after three weeks when the flies were held at 10 C and raised to 25 C every other day for 6 to 8 hours. When the flies were held at 27 C virus was recovered for 3 days from the excreta and for 5 to 12 days from the flies.

There does not appear to be any record of such experiments with cockroaches. The habits of these insects suggest that they might possibly be involved in the epidemiology of the disease. The present tests were undertaken to determine whether the mouse-adapted Lansing strain of the virus would persist longer if inoculated directly into the insect. Both houseflies and cockroaches were used.

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METHODS

A 10% emulsion of infected mouse spinal cord was inoculated into the hemocele of the housefly, *Musca domestica*, and the German cockroach, *Blatella germanica*, using a 0.25 cc syringe and a 27-gauge needle. The flies were given 0.005 cc each through the midline of the mesonotum, and the cockroaches 0.01 cc through the ventral intersegmental membrane at about the level of the 5th abdominal segment.

The insects were kept at a constant temperature of 30 C. On the 12th day, the 30 surviving flies were emulsified in 3 cc of physiological salt solution by grinding in a mortar with alundum. The emulsion was centrifuged lightly for 10 minutes, and the supernatant was then shaken with 40% ether by volume for 10 minutes to kill the bacteria. The ether was allowed to evaporate, and then 0.03 cc was inoculated intracerebrally into each of 8 mice. The vomit and fecal spots which had accumulated from these flies in a clean jar during the final 24 hours were removed with a cotton swab soaked with 50% normal rabbit serum and tested in a similar manner. In like manner also the 22 cockroaches surviving on the 15th day, 5 egg capsules dropped at this time and the feces which accumulated in a clean jar during the final 24 hours were emulsified and tested. Most of the cockroaches were females which were either gravid or had recently dropped their egg capsules.

RESULTS

The whole cockroach and egg suspensions were bacterially sterile in fluid thioglycollate medium, but the other three preparations produced some bacterial growth in this medium. The inoculated

TABLE 1.—*Recovery of poliomyelitis virus from cockroaches and houseflies.*

Insect material	Extrinsic period	1st mouse passage	2nd mouse passage
Whole roach	15 days	4/8*	8/8
Roach eggs	15 days	0/8	
Roach feces	15 days	0/8	
Whole fly	12 days	2/5	8/8
Fly vomit and excreta	12 days	0/8	

* Four of 8 mice inoculated developed paralysis in 30 days.

mice did not appear to be adversely affected.

The results are summarized in table 1. Four of 8 mice inoculated with the whole cockroach suspension developed paralysis of the forelimbs between the 13th and the 24th days. A 10% suspension of the cord and medulla of one of these was inoculated into the cerebrum of 8 normal mice, all of which developed typical paralysis and died in 4 to 14 days. The inoculum was bacterially sterile in fluid thioglycollate medium. The cervical portion of the cord was examined histologically and typical lesions were present, although not in large numbers. Two of 5 mice inoculated with the whole fly suspension developed paralysis on the 14th and 26th days, respectively. A 10% suspension of the cord and medulla of one of these produced typical paralysis in all of 8 mice inoculated, in from 3 to 19 days. The inoculum was bacterially sterile in fluid thioglycollate medium. A portion of the cord was examined histologically, and typical lesions were found to be present. All of the mice inoculated with emulsions of cockroach eggs, cockroach feces, and fly vomit and excreta material remained normal throughout the observational period of 30 days. These negative results may not be significant since only a small amount of material was tested.

The fly and roach suspensions represented dilutions of the original infected mouse cord of approximately 1:250. A suspension of fresh cord material in this dilution would ordinarily paralyze and kill, in from 2 to 30 days, all mice inoculated with it intracerebrally. For this reason, it seems likely that there was an actual decrease in the amount of virus in the insects during the experiment. However, the retention of active virus for as long as two weeks at 30 C is perhaps significant since in all tests previously reported at comparable temperatures, the virus of the mouse-adapted Lansing strain could not be recovered after 48 hours when it was fed to flies and was presumably present only in the gut or on the external surface of the body.

SUMMARY

1. Virus of the mouse-adapted Lansing strain of poliomyelitis was inoculated into the hemocele of German cockroaches and houseflies to determine whether the virus would remain active for an appreciable period of time under these conditions.

2. Emulsions of the bodies of the cockroaches and flies surviving for 15 and 12 days, respectively, produced typical paralysis in mice when inoculated intracerebrally.